New Series Vol. XVII No. 67 July 1953

Price 20*s*. net

ANNALS OF BOTANY

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OXFORD : AT THE CLARENDON PRESS

LONDON : GEOFFREY CUMBERLEGE

PRINTED IN GREAT BRITAIN
BY CHARLES BATEY AT THE UNIVERSITY PRESS, OXFORD

1953

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The Carbohydrate Nutrition of Tomato Roots

IV. The Nature and Distribution of Acid Phosphatases

BY

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AND

H. E. STREET

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With Plate XXI

ABSTRACT

The distribution of acid-phosphatase activity in sections of embedded, excised, and seedling roots has been studied by the Gomori technique. Evidence has been obtained for the presence of two orthophosphatases and of a pyrophosphatase. The results are discussed in the light of the possible roles of these enzymes in carbohydrate metabolism.

INTRODUCTION

MOST histochemical investigations of phosphatase activity have been carried out on animal tissues. Glick and Fisher (1945, 1946) have studied the distribution of acid-phosphatase in germinating wheat grain using a modification of the method of Gomori (1941), and Yin (1945) has investigated phosphatase distribution in plants of *Lamium* and *Iris*, using the technique of Menten, Junge, and Green (1944).

Yin found phosphatase activity to be highest in meristematic tissues, phloem elements, and chlorophyllous cells. In roots, the high activity of the meristem was maintained through the zone of primary differentiation in the procambial tissues but the activity of the cortical cells decreased markedly. In mature root tissues activity was generally slight, phloem elements being most active. Yin interpreted the high activity of the phloem as showing the association of phosphatase activity in the phloem parenchyma and companion cells with ability to accumulate and secrete sugars and thereby to maintain a concentration gradient of sugar in the sieve tubes. This interpretation is consistent with the evidence reviewed in earlier papers of this series (Dormer and Street, 1949; Street and Lowe, 1950) that cells active in sugar absorption and secretion have high phosphatase activity. It has been postulated that these processes involve the formation and breakdown of sugar phosphates and that the dephosphorylation stage is controlled by phosphatases (Höber, 1946; Dixon, 1949). Evidence has been presented that a phosphorylating mechanism, operating at the root surface, is involved in the absorption of

[Annals of Botany, N.S. Vol. XVII, No. 67, July, 1953.]

sucrose by excised tomato roots (Dormer and Street, 1949; Street and Lowe, 1950).

Excised roots might, therefore, be expected to show high phosphatase activity not only in the phloem as is the case in roots of intact plants but also in the cortex through which sugar is translocated from the external medium.

Experimental Procedures

1. *Root material*

The following types of root material were used:

(*a*) 7-day excised tomato roots, obtained by growth of 10-mm. root-tip inocula from a clone of tomato roots ('Best-of-All') for 7 days in White's medium, modified as previously described (Street and McGregor, 1952). These roots were approximately 90 mm. in length.

(*b*) 5-day excised tomato roots, obtained as in (*a*) above by 5 days' culture. The roots were 60–80 mm. in length.

(*c*) Seedling roots. Tomato seeds ('Best-of-All') were sterilized with 0·5 per cent. bromine water (Boll and Street, 1951) and germinated in a thin layer of sterile sand supported by several thicknesses of cotton gauze at the surface of a sterile culture solution consisting of the inorganic salts of modified White's medium at one-tenth of the normal strength. The radicles grew through the sand into the culture solution and developed laterals. Lateral roots 120–240 mm. in length, were harvested when the seedlings had grown in the light for 3 weeks.

(*d*) Radicles. Tomato seeds ('Best-of-All') were germinated on moist filter-paper in Petri dishes for 6 days. The radicles were about 35 mm. in length when harvested.

Excised roots and radicles were grown at $27 \pm 0{\cdot}5$°C., seedling roots at room temperature.

2. *Preparation of sections and methods of comparison*

All roots were fixed in 70 per cent. alcohol and embedded by the technique developed by Glick and Fisher (1945).

(*a*) Distribution of phosphatase activity in excised roots: Both transverse and longitudinal sections, 8μ in thickness, were prepared. For comparison of the distribution of activity along any one root the numbered root pieces were treated simultaneously during fixation, dehydration, embedding, and demonstration of phosphatase activity.

(*b*) Comparison of phosphatase activity in roots of diverse origins: For comparing seedling roots and 7-day excised roots, longitudinal sections were used. Each root was cut into 5 pieces as follows: a 20-mm. length (Part I) which included the meristem (I*a*—the apical 10 mm. and I*b*) and four equal lengths (Parts II–V numbered from the apex) constituting the remainder of the root after discarding the basal 10 mm. representing the original inoculum. Corresponding

parts from a seedling and an excised root were fixed and embedded together, sectioned separately, and the sections from both mounted side by side on the same slide. The six slides resulting from each pair of roots were treated together in a staining-bath during the demonstration of phosphatase activity.

The distribution of phosphatase activity along the axis of the radicles and 5-day excised roots was compared by a procedure similar to that described above.

3. *Demonstration of phosphatase activity*

Acid phosphatase activity has been demonstrated by the Gomori technique (Gomori, 1941) as modified by Glick and Fisher (1945). This involves the incubation of sections in a buffered solution of a phosphate ester and a lead salt ('substrate mixture'). Phosphate ions liberated by phosphatase action are precipitated as lead phosphate. Sites of activity are subsequently made visible by conversion of lead phosphate to black lead sulphide using a solution of ammonium polysulphide.

Sodium glycerophosphate has been used as substrate in investigating the distribution of acid phosphatase in excised and seedling roots. In the preliminary work substrate mixtures were prepared according to Gomori's original formula (Gomori, 1941), but this failed to give consistent results and subsequently mixtures were prepared according to Gomori's revised formula (Gomori, 1950).

In order to remove any preformed phosphate deposits sections were always treated with citrate buffer of pH 4·8 for 15 minutes prior to incubation (Gomori, 1946), although sections of root-tips treated first with 'substrate mixture' from which sodium glycerophosphate had been omitted and then with ammonium polysulphide, showed no precipitate of lead sulphide and, therefore, were apparently free from phosphate deposits.

Unless otherwise stated an incubation period of 2 hours at 41° C. has been employed. This is the maximum period for which sections can be incubated without the structure of the tissues becoming obscured at sites of high activity.

In all determinations of phosphatase activity the following two control treatments were included: (i) sections were placed in boiling water for 4 minutes after treatment with citrate buffer and before incubation; (ii) sections were incubated in 'substrate mixture' from which the substrate had been omitted. In all sections submitted to either of these control treatments no deposits of lead sulphide were visible.

Activity in longitudinal sections of tips of 7-day excised roots has also been studied using thiamine pyrophosphate (cocarboxylase) and glucose–1–phosphate as substrates. Substrate mixtures were prepared according to the formulae of Glick and Fisher (1945).

4. *Effect of pH and of fluoride on phosphatase activity*

The effect of pH on phosphatase activity in sections of excised roots has been studied over the range of pH 3·0—9·5, using sodium glycerophosphate

as substrate. Acetate (Vogel, 1945) and barbital-acetate buffers (Michaelis, 1931) were used. At pH values of 6 and below a lead salt was included in the substrate mixture as described above, but at higher pH values this was replaced by a calcium salt and sections were treated by the Gomori technique for the demonstration of alkaline phosphatase (Glick, 1949). No phosphatase activity could be detected at pH values above 5·2. Using acetate buffer positive results were obtained over the range pH 4·3–5·2 with maximum activity at pH 4·6–4·8. With barbital-acetate buffer activity occurred over the range pH 3·6–4·9 with maximum activity at pH 4·0–4·3. In all subsequent determinations of phosphatase activity acetate buffer of pH 4·8 was used in preparing substrate mixtures.

When sodium fluoride was added to the substrate mixture to give a final concentration of M/100, phosphatase activity was markedly reduced. With incubation periods of up to 6 hours a positive reaction occurred only in the nuclei.

Experimental Results

1. *Detailed study of acid phosphatase distribution in the tissues of 7-day excised roots*

Phosphatase activity occurs in the nuclei. In interphase nuclei, activity is centred in the nucleoli and heterochromatic regions at the periphery; in dividing nuclei it occurs in the chromosomes (Pl. XXI, Fig. 1). Activity occurs at or in the walls of meristematic and differentiating cells. In exodermal cells, activity is frequently confined to the radial walls. Differentiating xylem vessels show activity but mature vessels are inactive. In the cytoplasm the intensity of phosphatase activity is characteristic of the various tissues and alters as their cells differentiate. The level of extra-nuclear activity has been used as the criterion for the assessment of the relative activity of the various tissues as described below.

(*a*) Meristem and root-cap: This zone shows the highest activity. Deposition of lead sulphide is most intense in the root-cap, particularly in its outermost cells. Activity is almost as high in the cortical meristematic cells. By contrast the stelar cells are inactive or show very low activity (Pl. XXI, Fig. 3). A similar distribution of activity occurs in the apices of lateral roots (Pl. XXI, Fig. 4).

(*b*) Zone of cell elongation. During cell elongation stelar cells are active (Pl. XXI, Fig. 2) so that within the stele a dark zone occurs between the much less active regions of the meristem and mature primary tissues. Activity decreases as cell elongation proceeds and in the cortex falls markedly as cell elongation reaches completion. The decrease in activity occurring after cell expansion was judged to be real and not merely a reflection of the changing ratio of cytoplasmic content to cross-sectional area of the cells.

(*c*) Mature tissues: Only a few cells of the piliferous layer show activity and their number is not increased by lengthening the incubation period beyond the standard period of 2 hours. Similarly, exodermal cells are

frequently inactive; where activity does occur it is usually confined to the radial walls.

The outermost layer of cortical parenchyma, immediately within the exodermis, usually shows intense activity resulting in a dark band against the inner tangential wall of the exodermal cells (Pl. XXI, Fig. 3). In the cortical cells internal to this layer the deposition of lead sulphide is much less intense or absent. Extension of the incubation period beyond the standard period of 2 hours, however, always shows these cells to possess some activity. The endodermis is usually more active than the inner cortical parenchyma. The comparatively low activity of the cortex described above characterizes the region extending from the zone of elongation into the zone of lateral root initiation (Pl. XXI, Fig. 4). In the older parts of the root there is an increase in the phosphatase activity of the cortex (Pl. XXI, Fig. 3).

The pericyclic cells show activity and a slight enhancement of this activity occurs where cell-division is taking place to form lateral root initials. Mature xylem vessels are inactive. All phloem cells are active, protophloem cells showing least activity. The highest activity in the stele occurs in the conjunctive parenchyma in which a cambial band is known to arise (Dormer and Street, 1948).

2. *Comparison of the phosphatase activity of excised roots with that of seedling roots and radicles*

The anatomy of the seedling roots and radicles was similar to that previously described for excised roots (Dormer and Street, 1949; Street and McGregor, 1952). The radicles had not produced laterals when harvested and the seedling roots had developed few laterals as compared with excised roots. The general distribution of phosphatase activity in seedling roots and radicles agreed with that described above for excised roots. The intensity of phosphatase activity immediately behind the zone of elongation was very low in excised roots, seedling roots, and radicles. In both the seedling roots and radicles a similar low level of activity characterized the older parts of the root. By contrast in excised roots phosphatase activity increased some 20–40 mm. from the apex and became progressively more intense with increase in distance from the apex (Pl. XXI, Figs. 3 and 4). This increase in activity usually occured in all tissues, though in some roots it was not marked in the inner cortex. Estimated levels of phosphatase activity in specimen comparisons between excised roots and seedling roots are shown in Table I.

3. *Demonstration of the acid phosphatase activity of tips of excised roots using sodium glycerophosphate, glucose-1-phosphate and thiamine pyrophosphate as substrates*

The distribution of lead sulphide deposits with glucose-1-phosphate resembles that obtained with sodium glycerophosphate. The activity with glucose-1-phosphate is, however, much lower. With thiamine pyrophosphate (cocarboxylase) the whole of the meristem and zone of elongation

shows high activity which is at a maximum in the root-cap and zone of stelar cell elongation (Pl. XXI, Fig. 5). The high activity of the stelar meristematic cells with thiamine pyrophosphate is in marked contrast to the results obtained with the other two substrates. Furthermore, with thiamine pyrophosphate the nuclei are unstained.

TABLE I

Comparison of the Intensity of Phosphatase Activity in Corresponding Zones of Excised (E) and Seedling Roots (S). Intensity grading for any one comparison:

$$++ > +(+) > + > (+)$$

		Specimen comparisons					
	Part of root	E	S	E	S	E	S
I *a*	Meristem	++++	++++	+++	+++	+++	++++
	Zone of Elongation	++	++			++	+++
	Remainder	+	+(+)			+	++
I *b*		+	+(+)	nil	++(+)	+	+
II		+	+(+)	(+)	++(+)	++	++
III		(no section)	+(+)	+(+)	++	++	(+)
IV		+++	+(+)	++	nil	++(+)	(+)
V		+++	+(+)	++	nil	+++	(+)

DISCUSSION

Macdonald (1950), using animal tissues, has shown that the nucleus, and particularly the nucleolus, may have an affinity for lead nitrate, glycerophosphate, and phosphate ions, and she has, therefore, concluded that a positive intra-nuclear Gomori reaction is not necessarily indicative of the presence of phosphatase. In our 'substrate-omitted' controls the nuclei were inactive and, therefore, the positive reaction cannot result from an affinity of the nuclei for lead nitrate. Furthermore, no activity occurred when heat-treated sections were incubated with substrate. These considerations and the fact that activity in the presence of fluoride was concentrated in the nucleus support the view that the nuclear activity of our material results from the presence of an acid phosphatase insensitive to fluoride. Novikoff (1951) has, however, shown that considerable deposition of calcium phosphate occurs in the nuclei of rat liver treated by the technique for alkaline phosphatase, although the phosphatase activity of the nuclei is low as determined by direct biochemical assay. This raises the possibility that diffusion of phosphatase or lead phosphate from the cytoplasm to the nucleus may occur at some stage of the technique. Such diffusion might account for the apparent high nuclear activity but not for the difference in sensitivity to fluoride of cytoplasmic and nuclear activity.

Macdonald has also raised the question of movement of enzyme or reaction products between tissues at some stage of the technique. She observed such movement during prolonged incubation at low temperature. The adoption

of a 2-hour incubation period and the sharply defined contrasts in activity between adjacent tissues in our phosphatase-distribution pictures both make it improbable that any significant redistribution of phosphatase enzymes has occurred in the present investigation. The activity noted, in some cases, in the stelar tissues immediately adjoining the lateral root initials may, however, result from such a movement since elsewhere in the zone of lateral root initiation the activity of the stele is very slight (Pl. XXI, Fig. 4).

From the experimental results presented it can be tentatively suggested that three acid phosphatases occur in excised tomato roots: (i) an orthophosphatase, active over the range pH 4·3–5·2, inhibited by fluoride and occurring in the cytoplasm; (ii) an orthophosphatase, active at acid pH, insensitive to fluoride, and concentrated in the nucleus; (iii) a pyrophosphatase, active at acid pH, confined to the cytoplasm, and having a different distribution in the root-apex from that of orthophosphatase (i). The behaviour of the orthophosphatases corresponds with that of Types II and III of the phosphomonoesterases of Roche's classification (Roche, 1950).

Our failure to demonstrate alkaline phosphatase activity in tomato roots may be due to the use of sodium glycerophosphate as the test substrate. Ross and Ely (1951), using the Gomori technique, have demonstrated in the nuclei of embedded root-tips of onion and maize the presence of alkaline phosphatase unable to utilize sodium glycerophosphate but active with several other substrates.

The localization of acid phosphatase activity in the various tissues of tomato roots agrees in general with that found by Glick and Fisher (1945, 1946) in wheat radicles and by Yin (1945) in roots of *Lamium* and *Iris*. In all cases activity was at a maximum in the meristem and in the zone of primary cell differentiation. In both tomato and wheat roots high activity occurred in the root-cap. By contrast Yin found no activity in the root-cap cells of the species which he investigated. The root-cap cells in tomato contain abundant starch grains and the high phosphatase activity may be associated with starch synthesis. Yin found acid phosphatase activity in the starch-storing cells of the potato tuber and of the *Iris* rhizome. Wislocki and Dempsey (1945) found high alkaline phosphatase activity at sites of glycogen synthesis in animal tissues. The observation that, in tomato roots, phosphatase activity also regularly occurs in cells where cell-wall material is being synthesized supports the view that phosphatases may be involved in polysaccharide synthesis.

Yin reported that behind the zone of primary cell differentiation there was a die-away of phosphatase activity in the cortex. This agrees with the distribution of phosphatase activity found in seedling tomato roots but contrasts with that in excised roots. In excised roots the cortical meristematic cells have high activity, this activity decreases as cell elongation proceeds, is at a very low level in the region of lateral root initiation, begins to increase some 20–40 mm. from the apex, and then becomes progressively more intense. The repeatedly observed occurrence of high phosphatase activity in cells effecting solute transfer suggests that this renewal of phosphatase activity in

the mature cortical cells may be indicative of their activity in sugar absorption. Excised tomato root-tips 10 mm. in length when transferred to culture solutions show, during the first 24–48 hours of culture, a relatively slow rate of growth which when the root is some 20–30 mm. long is followed by a period of rapid linear growth rate which continues until checked by 'staling' of the medium (Street, McGonagle, and Lowe, 1951). The lag-phase can be prolonged by using 5-mm. tips, but is not to be observed if 30-mm. inocula are used (Table II). The low initial growth rate of short inocula may be due to a greater traumatic effect from excision. However, it may be that the rise in growth rate of the excised root is due to an increased ability to absorb sugar as the cortical cells mature and develop phosphatase activity.

TABLE II

Influence of the Length of Excised Tomato Roots on their Linear Growth Rate in Modified White's Medium. Inocula from 7-day sector cultures of the clone, 'Best-of-All'

Inoculum length (mm.)		Period (hrs.) 0–24	24–48	48–72	72–96	96–108	No. of cultures
4·5	Mean root length (mm.) during period	5·7	9·8	16·8	26·9	37·7	18
	Increase in length (mm.) during period	2·4±0·8*	5·8±0·5	8·4±0·5	11·5±0·5	9·2±0·6	
10·0	Mean root length (mm.) during period	12·2	18·2	26·6	36·9	47·2	20
	Increase in length (mm.) during period	4·4±0·3	7·5±0·5	9·4±0·4	11·3±0·7	9·5±0·5	
30·0	Mean root length (mm.) during period	35·2	46·6	59·7	71·9		19
	Increase in length (mm.) during period	10·5±0·9	12·7±0·8	13·7±0·7	10·7±0·3		

* Standard error.

Cortical cells in the original inoculum portion of the root and in the oldest parts of the new growth frequently contain starch grains. The high phosphatase activity of these cells may, therefore, be related to starch synthesis. The renewal of phosphatase activity behind the zone of lateral root initiation, however, clearly takes place in cells not synthesizing starch. It is, therefore, possible that starch synthesis may be a secondary activity occurring as the older cortical cells, now no longer acting as part of the channel of sugar transport to the meristematic tissues, become increasingly rich in sugar. Yao (1950) in his study of post-embryonic development of *Drosophila* found formation of both alkaline and acid phosphatase to occur during histolysis of each larval organ. The hypothesis that the high phosphatase activity of the root-cap and of the older regions of the excised root is indicative of cellular degeneration in these tissues must be considered. However, against such an interpretation are the facts that there is little or no phosphatase activity in degenerating piliferous layer cells and that there is no histological evidence of lysis in the older regions of the root.

Summary

1. The distribution of acid phosphatase activity in sections of embedded excised roots and seedling roots of tomato has been determined by the Gomori technique using sodium glycerophosphate as substrate.

2. Activity occurred over the pH range 4·3–5·2 (acetate buffer) with maximum activity at pH 4·6–4·8. M/100 sodium fluoride inhibited activity in the cytoplasm.

3. In the root apex, maximum activity occurred in the root-cap and extrastelar meristematic cells. Activity in the stelar meristematic cells was low except in the nuclei. In mature tissues maximum activity occurred in the phloem and conjunctive parenchyma and in the cortical layer immediately within the exodermis. The piliferous layer was of low activity. A positive response occurred at the walls of differentiating xylem vessels, exodermal cells, and meristematic cells. Mature xylem vessels were inactive.

4. The intensity of activity varied with distance from the apex. The extrastelar activity was at a maximum in the meristem, decreased in the zone of elongation, and became very low in the zone of lateral root initiation except in the initials themselves which were highly active. Stelar activity which is low in the meristem increased at the beginning of the zone of elongation, decreased as elongation reached completion and was low in the zone of lateral root initiation. In excised roots the activity in both stele and cortex increased behind the zone of lateral root initiation whilst in seedling roots it became further reduced.

5. Using glucose–1–phosphate as substrate the distribution of activity in excised root-tips resembled that obtained with glycerophosphate but the activity was lower. Using thiamine pyrophosphate the stelar meristematic cells were highly active and no intra-nuclear reaction occurred.

6. The studies of phosphatase distribution and the results with fluoride point to the probable presence in tomato roots of two orthophosphatases and a pyrophosphatase.

7. The results are discussed in the light of the possible roles of phosphatases in carbohydrate metabolism.

Acknowledgements

Grateful acknowledgement is made to Mr. P. Chambers for the photographs in Pl. XXI and to the Agricultural Research Council for a grant which has enabled one of us (S. M. McGregor) to take part in this work.

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EXPLANATION OF PLATE

Illustrating S. M. McGregor and H. E. Street's paper on 'The Carbohydrate Nutrition of Tomato Roots', IV.

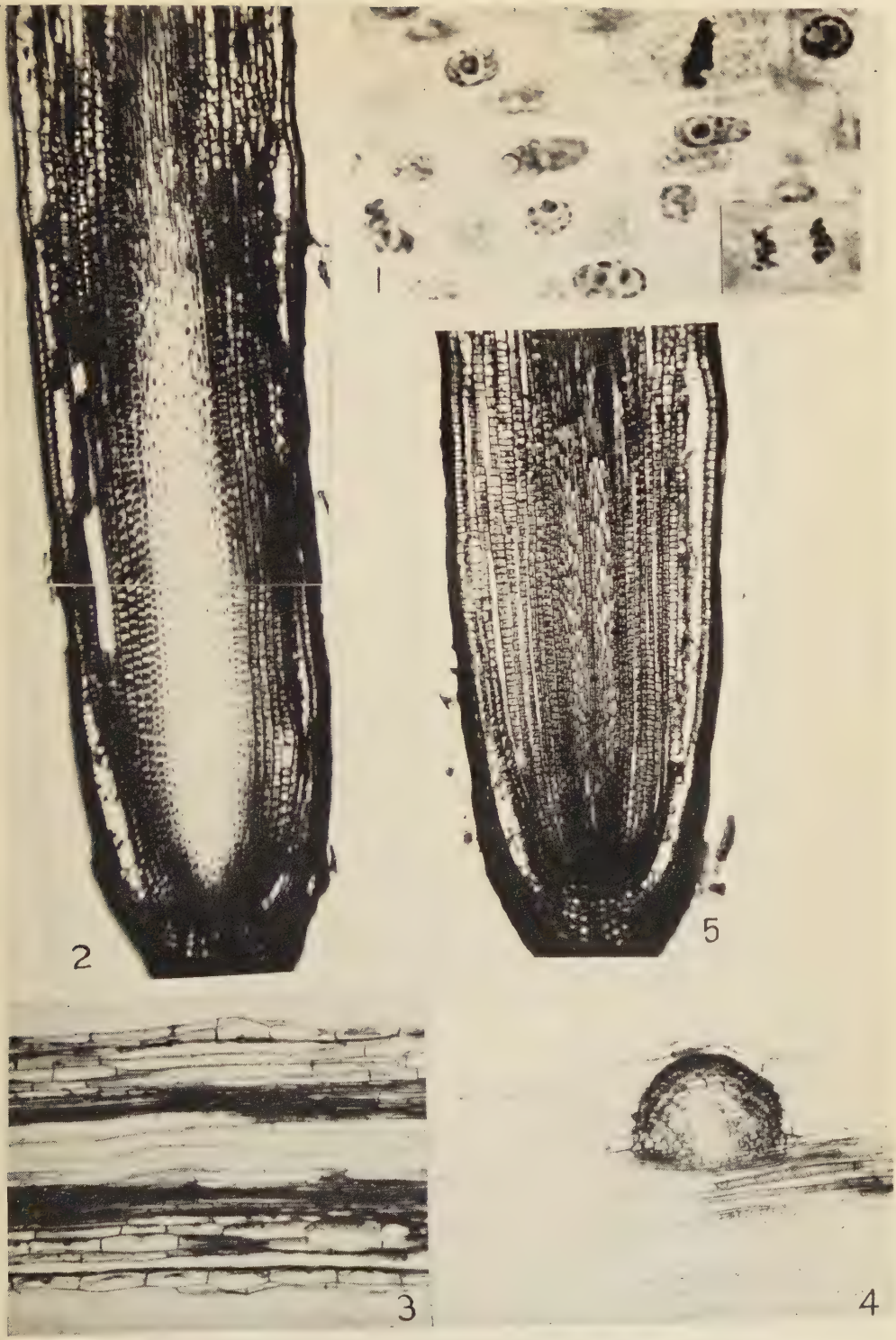
Distribution of acid phosphatase activity as seen in longitudinal sections of 7-day excised roots. Figs. 1–4 using sodium glycerophosphate; Fig. 5 using thiamine pyrophosphate as substrate.

Fig. 1. Intra-cellular distribution in stelar meristematic cells.

Figs. 2 and 5. Root apex.

Fig. 3. 45–50 mm. from apex.

Fig. 4. 15 mm. from apex.



S. M. McGREGOR and H. E. STREET

Experimental and Analytical Studies of Pteridophytes

XXI. Sterile cultures of an abnormal growth from the gametophyte of bracken

BY

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AND

TAYLOR A. STEEVES[1]

With Plate XXII

Abstract

The isolation and growth in sterile culture through sixteen passages, each of 4 to 6 weeks' duration, of an abnormal growth isolated from a gametophyte of *Pteridium aquilinum* var. *latiusculum* is reported, and the nutritional requirements described.

The cultures consist of green, photosynthetic, predominantly filamentous, but coherent, masses. Prothalli were regenerated on five occasions during the first two passages only. Outgrowths of whitish or pale green parenchymatous tissue occur. These possess a central strand of elongated cells, some of which become differentiated into a core of tracheides.

Prothalli of the strain in culture are haploid. When examined soon after the initial explantation, the filamentous parts of the cultures were diploid, but there has been a subsequent increase in the number of chromosomes, and each cell is now irregularly aneuploid, with a chromosome number between triploid and tetraploid.

Introduction

In contrast to the numerous cultures of callus tissues which have been isolated from phanerogams, *in vitro* cultures of tissues of vascular cryptogams have been obtained on few occasions only. The first accounts of such cultures were given by Morel and Wetmore (Morel, 1950; Morel and Wetmore, 1951), who reported the spontaneous appearance on several prothalli of *Osmunda cinnamomea* growing in sterile culture of undifferentiated parenchymatous calluses which, when subcultured on to an agar medium of inorganic salts, sugar, and B vitamins, were capable of potentially unlimited growth. Wetmore and Morel have obtained further callus cultures from gametophytes of *Lycopodium cernuum* (1951*a*) and from megagametophytes of two species of *Selaginella* (1951*b*). Morel has successfully subcultured callus from the cut surfaces of stem tips of *Selaginella wildenovii* growing in

[1] Society of Fellows of Harvard University, U.S.A.

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sterile culture (personal communication), and from sporophytes of *Osmunda cinnamomea* (1950), but these latter were not capable of unlimited growth on the media used.

In the present paper the isolation and growth in sterile culture of an abnormal growth derived from the gametophyte of the bracken fern, *Pteridium aquilinum* (L) Kuhn, var. *latiusculum* (Desv.) Underw. (Tryon, 1941), is reported, together with a preliminary morphological, anatomical, and cytological description of the cultures so derived.

Origin and Maintenance of the Cultures

In July 1950 gametophytes of *Pteridium aquilinum* var. *latiusculum*, growing in sterile culture at Harvard University, U.S.A., were transferred to a 1 per cent. agar medium containing Knudson's solution of inorganic salts (1925), Berthelot's trace element solution, 2 per cent. glucose, and 0·5 g./l. yeast extract (autoclaved). In October of the same year, after the cultures had been moved to Manchester, England, a green, callus-like growth was observed in one tube. The proliferation appeared to be an outgrowth from the surface of a prothallus, but because of the advanced stage of development it was not possible to determine its origin with certainty.

This abnormal growth was subcultured on to the same medium, with half-strength Knop's solution (Gautheret, 1942) substituted for Knudson's, on which it has given every indication of potentially unlimited growth. It has now been through sixteen passages, each of 4 to 6 weeks' duration, and in each passage there has been a six- to ten-fold increase in the size of the inoculum.

Some cultures have been maintained in daylight, but most have been kept in a room with the temperature regulated at $21° \pm 1°$ C., and with a single 'daylight' fluorescent lamp (5 ft., 80 W) suspended 18 in. above the cultures as the sole light source.

Morphology and Anatomy

The cultures are deep green in colour and, although forming coherent masses are friable and easily fragmented with a needle to provide explants suitable for subculturing. When first subcultured, the surfaces of the cultures are moist and glistening, but they gradually become dull and velvety through the outgrowth of short filaments. This change in appearance with age is well shown in Pl. XXII, Fig. 1. On the left is a culture 23 days after subculturing, while that on the right has grown for 34 days. It will be noted that growth takes place from several distinct foci and this causes the surface of the cultures to be thrown into irregular mounds and depressions.

Examination of the microscopic structure of the cultures shows them to be predominantly filamentous. Numerous branched filaments radiate from common centres, each of which appears to be an independently growing unit. One such centre appears in Pl. XXII, Fig. 2. Several of the radiating filaments have been sectioned longitudinally throughout much of their length, and from

this figure, together with Pl. XXII, Fig. 3, in which is shown a single filament dissected out from a culture, a clear picture of the method of growth may be obtained. Within each filament divisions at the tip are restricted to the transverse plane, the terminal cell functioning as an apical cell. Some little distance behind the tip of the filament divisions occur in other planes. In occasional filaments these subsequent divisions are restricted to the transverse and longitudinal vertical planes so that flat, broad filaments, a single cell in thickness, are formed; in most, divisions occur in any plane and result in the development of a more or less extensive region of tissue in older parts of the filament. From superficial cells of these thicker regions, secondary filaments arise (Pl. XXII, Fig. 3) and by the death of cells in the centre of the original growth unit, each primary filament may itself become a new centre of growth (Pl. XXII, Figs. 2 and 3).

In some regions which appear to be truly parenchymatous the cells are large and highly vacuolated (Pl. XXII, Fig. 4). The origin of these regions has not been elucidated, but they may result from the continued division in various planes of a filament of cells.

The cells of the filaments are spherical or ovoid and each contains numerous small chloroplasts which are either distributed in the periphery of the cell or aggregated around the nucleus. In old cultures grown on an agar medium some cells grow out as short, branched, aseptate rhizoids devoid of chloroplasts.

In many of the cultures whitish or pale green protuberant outgrowths project beyond the surface. A group of these appears on the right in Pl. XXII, Fig. 5, and contrasts with the filamentous portions of the cultures at the left of the figure. Sections show that the protuberances are parenchymatous tissues of small, deeply staining cells (Pl. XXII, Fig. 6). In them, growth is largely confined to certain localized meristematic regions of the surface, in which the segmentation frequently resembles that found in the shoot apex of young sporophytes. Behind the more rapidly growing of these 'meristems' there is often a median strand of rather elongated narrow cells, some of which may be further differentiated into a continuous core of short scalariform or reticulate tracheides. No phloem elements have been observed in these strands.

In five tubes of the first two subcultures, but in no later ones, regeneration of apparently normal prothalli occurred. Since the regeneration took place on several different media, it has not been possible to determine the factors responsible; nor has it been possible to induce further prothallial regeneration in later subcultures although a wide variety of media has been used. Morel and Wetmore (1951) noted a similar initial regenerative capacity, which was soon lost, in *Osmunda* callus.

Cytology

No records of the chromosome number of *Pteridium aquilinum* in America are available, but from material collected in Britain and in Malaya, Manton (1950) has reported a haploid number of 52.

Throughout the present investigation the acetocarmine squash method has been used to obtain stained preparations for examination. On a few occasions the material was pretreated with colchicine or with paradichlorbenzene, but this was found to be unnecessary, and during most of the investigation this pretreatment was omitted.

Prothalli of the strain from which the cultures were isolated were found to have a chromosome number of approximately 50, which agrees closely with the haploid number recorded by Manton. It appears, therefore, that the abnormal growth originated from haploid tissue. When first examined cytologically the *Pteridium* cultures were in the fourth transfer, about 4 months after the initial explantation. At that time a few chromosome counts were made and the number obtained in each case was approximately 100. It was concluded that the cultures were diploid. When a further, and more detailed, investigation was made of material in the twelfth and fourteenth transfers, the numbers obtained were considerably higher, ranging from approximately 150 to approximately 200. No cell was found in which the number was exactly tetraploid, i.e. 208. Preparations such as that shown in Pl. XXII, Fig. 7, are sufficiently clear that, despite the very large numbers of chromosomes present, it is thought that each count is accurate to within four or five chromosomes. It is apparent that not only has there been an increase in the chromosome number during the maintenance of the cultures, but that chromosome multiplication has proceeded at different rates in different cells. The filamentous regions of the cultures, which are the only parts to have been investigated cytologically, are therefore irregularly aneuploid.

Nutritional Requirements

Although the abnormal tissue of *Pteridium* arose on a medium containing yeast extract, it maintains active growth, without important morphological alterations on a simple medium consisting of Knudson's or Knop's solution of inorganic salts, micro-elements, and 1 per cent. glucose, solidified with 1 per cent. washed agar. No added growth factors are required. The ability of tissue cultures to maintain growth without added growth factors has been reported by Gautheret (1950) for carrot tissue grown for three years in the dark, and is a common phenomenon in cultures of crown gall and habituated tissue. It has been possible to grow the *Pteridium* cultures on an agar medium containing only inorganic salts. Growth on such a medium is very slow, but it does suggest that the cells of these abnormal tissues have retained the ability to synthesize organic nutrients. The cultures will grow submerged in liquid medium containing only inorganic salts and 3 per cent. glucose.

Discussion

Gametophytes of *Pteridium aquilinum* var. *latiusculum* have been grown in sterile culture at Harvard University for several years. In addition to the alusu plate-like prothalli, thickened prothalli have been observed, often associated

with the development of apogamous sporelings, and filamentous growths of various sorts, some of which resemble those observed by Hurel-Py (1951). None of these abnormal growths has yet proved capable of maintaining itself indefinitely on an inorganic medium.

The factors which were responsible for the original production of the filamentous growths described here are unknown. Attempts to reproduce such abnormalities by placing normal gametophytes of *Pteridium* on a medium of the same composition as that which produced the original have so far been unsuccessful. Abnormal growths, which superficially resemble the original cultures, can be induced, but when explanted, even on to a complex medium, these give rise to relatively normal, plate-like gametophytes. On the other hand, except for a few isolated cases in the first two transfers the original cultures do not produce gametophytes even when grown on a medium which usually supports normal prothallial growth.

The predominantly filamentous nature of these cultures is unusual, but not without parallel, in plant tissue culture. Gautheret (1942) in cambial cultures of *Salix* and in root slices of carrot, celery, and parsnip has reported that early proliferation from individual cells resulted in the production of branching filaments which he called 'pseudo-thalli'. Subsequent development, however, resulted in true callus tissues. De Ropp (1947) has obtained proliferations from stem cambium of *Helianthus*, in response to high auxin concentrations, in which the cells developed into loosely compacted elongated elements. Perhaps more comparable to the *Pteridium* cultures are those of *Parthenocissus tricuspidata* described by Morel (1948), in which the surfaces were covered by long files of cells joined into irregular bundles which were isolated from one another.

The parenchymatous outgrowths which project above the surfaces of the cultures resemble more the callus tissue cultures which have been derived from other vascular cryptogams. Although segmentation in the 'meristems' of these outgrowths is sometimes comparable to that found in the shoot apex of sporelings, there has, as yet, been no regeneration of sporophytic buds from them. By the presence of tracheides these outgrowths resemble the haploid callus of *Osmunda*, but whereas Morel and Wetmore (1951) state that the tracheides in their cultures are not associated with apogamy, the tracheide-containing outgrowths of the *Pteridium* cultures resemble an early but arrested stage of sporophytic bud development. By further culture under a variety of conditions it may be possible to determine whether these outgrowths are able to regenerate sporophytic buds, and the relationship between the presence of tracheides and the early stages of regeneration may then become apparent.

The greatly increased and irregularly aneuploid chromosome numbers of the cells of the *Pteridium* cultures provide an interesting parallel to the physiological abnormality which has been observed. There are apparently no cytological studies of comparable tissue cultures except that in *Osmunda* in which the callus was, like the gametophyte from which it arose, haploid. In crown

gall of beet and tobacco, however, Levine (1931) ofund, in addition to normal diploid cells, both tetraploid and octoploid cells. The diploid cells occur at the periphery; cells containing the higher chromosome numbers are restricted to the central portions. With one exception, he found no irregularities of chromosome numbers such as have been observed in the *Pteridium* cultures. Winge (1927) had previously concluded that crown gall of beet is composed essentially of tetraploid cells with occasionally occurring octoploid cells. In the *Pteridium* cultures, as in crown gall, however, it is not clear whether the cytological condition causes the physiological abnormality of the cells, or whether the reverse is true, but the increasing chromosome complement of the cells in successive transfers may be of particular importance in this connexion.

Acknowledgements

We wish to thank Professor C. W. Wardlaw for his encouragement and advice, and Professor R. H. Wetmore and Dr. G. Morel for many valuable suggestions. Our thanks are also due to the late Mr. A. Swaffer, to Dr. C. M. Wilson and Mr. C. Partenan, from each of whom we received considerable assistance during the cytological studies, and Mr. E. Ashby and Mr. P. T. Dawes for the majority of the photographic illustrations.

During the investigation one of us (T. A. S.) held a Sheldon Travelling Fellowship from Harvard University.

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DESCRIPTION OF PLATE

Illustrating I. M. Sussex and T. A. Steeve's paper on 'Experimental and Analytical Studies of Pteridophytes. XXI. Sterile cultures of an abnormal growth from the gametophyte of bracken'.

Fig. 1. External appearance of cultures. That on the left has grown for 23 days from subculturing, that on the right for 34 days. (×2.)

Fig. 2. Section of the culture showing a growth unit with radiating filaments, some of which have been sectioned longitudinally. Cells at the centre of the growth unit are dead. (×60.)

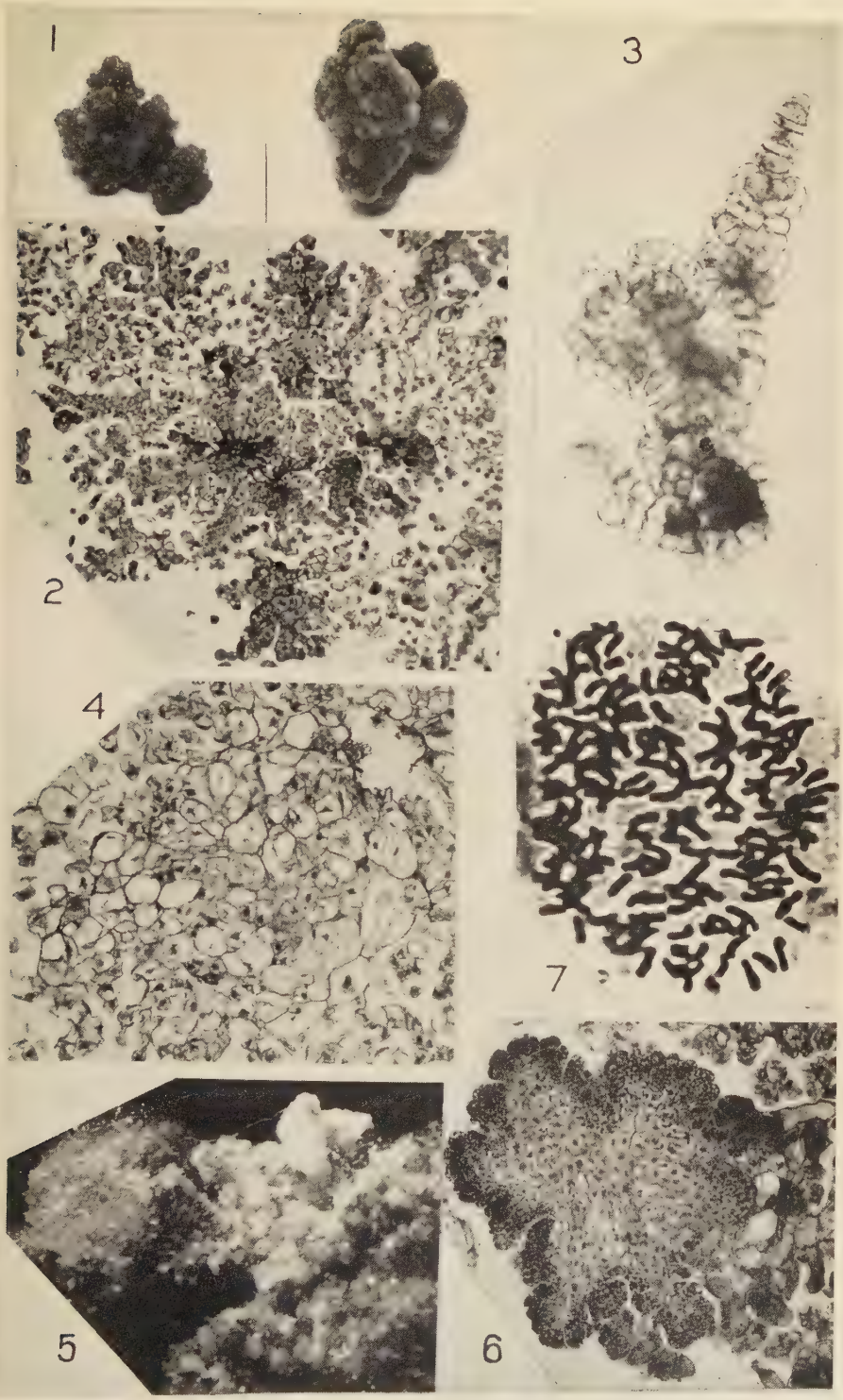
Fig. 3. A filament showing the uniseriate tip and thickened mature region with secondary filaments and dead cells from the centre of the growth unit. (×110.)

Fig. 4. Vacuolated parenchymatous cells which are found in the filamentous parts of the cultures. (×110.)

Fig. 5. Surface of a culture showing on the left a green filamentous region and on the right a group of paler protuberant outgrowths. (×6.)

Fig. 6. Section of a protuberant outgrowth showing its attachment to the filamentous cells. The surface cells of the figured outgrowth are filled with metabolic byproducts, the inner cells are parenchymatous, and some are elongated into a central vascular strand. (×110.)

Fig. 7. An acetocarmine preparation of metaphase chromosomes from a cell of a culture in its 14th transfer. 181 chromosomes were counted in this preparation. (×1,750.)



I. M. SUSSEX and T. A. STEEVES

Geitler's Nucleolar Substance in *Spirogyra*

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With Plates XXIII and XXIV and six Text-figures

Abstract

The movements of Geitler's 'nucleolar substance', a deeply staining material found in the mitotic nucleus after breakdown of the nucleolus, are traced in fifteen species of *Spirogyra* including two of those originally used.

Deposition of nucleolar substance on metaphase and anaphase chromosomes, noted by Geitler, is confirmed. Observed for the first time are the sloughing-off from the anaphase chromatids of nucleolar substance and its distribution in and by the spindle, independent of the chromosomes.

Introduction

GEITLER (1935) described the presence and distribution of the substance named by him 'nucleolar substance' in three species of *Spirogyra*, *S. setiformis*, *S. crassa*, and *S.* species *X*. In so doing he was the first to describe a type or rather types of mitosis unique among plants, in that a substance other than chromosome and spindle-fibre substance, and other than the nucleolus itself, is normally present although in varying amount and with varying distribution according to the species. In referring to this substance as 'nucleolar substance', because it looks like the nucleolus in *Spirogyra X* and can be seen to derive from breakdown of the nucleolus in *S. setiformis*, he has apparently given rise to the idea that the nucleolus itself persists during mitosis.

This confusion is well illustrated in the following quotations: 'the chromosomes [in *S. setiformis* and *S. X*.] secondarily migrate into the substance of the nucleolus'; '. . . in these two species the nucleolar material is persistent. . . . In other species however the nucleolus disappears in early prophase . . .'; '. . . the divisions [in *Lomentaria rosea*] occur within the nucleolus, in a fashion somewhat similar to that described by Geitler and Doraiswami for some species of *Spirogyra* . . .' (Bold, 1951, p. 216). Another author states: 'Thus, division of the chromosomes [of *Lomentaria rosea*] takes place within the nucleolus, just as in *Spirogyra*' (Svedelius, 1937, p. 13); and another, 'The chromosomes . . . are . . . within the nucleolar matrix. The nucleolar plate then splits transversely into two . . .' (Doraiswami, 1946, p. 23).

The tendency has been for authors to regard the 'nucleolar substance' and the nucleolus as identical, or as so much alike that the term 'nucleolus' could

[Annals of Botany, N.S. Vol. XVII, No. 67, July, 1953.]

upon occasion safely be used for both. This was not Geitler's view. Although in describing mitosis in *Spirogyra X* he differentiates the two terms merely by using 'nucleolus' for the resting nucleus and early prophase, 'nucleolar substance', for the later stages, in which his figures show (1935, p. 12) that there is little dispersal of the nucleolar material, his final remarks (1935, p. 18), which embody his conclusions about the distribution of the 'nucleolar substance' in the three species studied, also show that he is certainly not talking about the nucleolus. They are translated here in part: '. . . Comparison of the *Spirogyra* species shows that a substance is present which, considered purely topographically, appears, now as nucleolar substance, now as chromosome substance, or, in other words, shows that the closeness of the relationship to the chromosomes varies. In *crassa* the substance seems permanently attached to the chromosomes. In *S. setiformis* the loading of the chromosomes with substance during the anaphase can be directly followed.' He goes on to remark that *Spirogyra X* is the extreme type since at anaphase the chromosomes are invisible on account of the heavy investment of 'nucleolar substance'. Thus he establishes the remarkable fact that after the breakdown of the nucleolus a substance derived from it may remain in the nucleus, to be concentrated round the metaphase plate and to be carried with or on the chromatids to the telophase nucleus.

Since the publication of Geitler's and other accounts (Doraiswami, 1946) of nuclear division in *Spirogyra*, the writer has described the nucleolar organizing chromosomes of four species of the genus (Godward, 1950), including two of those referred to by Geitler (1935), namely *S. crassa* and *S. setiformis*. A considerable number of other species has also been investigated, their nuclear organizing chromosomes observed, and the distribution of the 'nucleolar substance' during mitosis recorded. As a result it is possible to make a somewhat wider survey of 'nucleolar substance' in relation to the chromosomes than that initiated by Geitler in 1935. This follows.

The Chemical Nature of the 'Nucleolar Substance'

According to Geitler (1935*a*) the nucleolar substance stains, like the nucleolus, with acetocarmine, not with Feulgen's stain; whereas the chromosomes or chromocentres, in some species at least, stain with Feulgen's stain. As described in the following pages, it is stained with iron–alum–acetocarmine (Godward, 1950). With pyronin and methyl green it stains with pyronin. It is partially opaque to ultra-violet light (2,750 Å), and an ultra-violet photograph, for example of anaphase, *S. crassa*, looks like the stained preparation. In all these respects it is like the nucleolus.

The Species used

Among the species of *Spirogyra* referred to here are three new species, viz. *S. brittannica, S. sub-echinata, S. sub-margaritata.* No description is given here. It is given in full in a separate publication (in preparation).

S. setiformis is the species of that name cytologically investigated by Van

Wisselingh and Geitler (see Godward, 1951). *S. triformis* is the species of that name described and cytologically investigated by Van Wisselingh (see Godward, 1950). *S. oblata* is the species cytologically investigated by the present author (1950) under the name, tentatively given on the basis of the vegetative characters, of *?ellipsospora*. Now that the zygospores have been found, this name has been shown to be incorrect.

Unidentified species are referred to by the following designations until such time as their zygospores have been recognized: *S. L.S.*; *S. 4.N.O.*; *S. M.1*; *S. F.*

Abbreviations, used mainly in figure-descriptions, are: N.O. = nucleolar organising, N.S. = nucleolar substance.

The work described here has been the subject of a note in 'Nature' (Godward, 1950*a*).

It will be convenient to describe in turn the breakdown of the nucleolus with the precipitation of the nucleolar substance into the nucleus; the distribution of the nucleolar substance in the nucleus at mid-prophase, particularly in relation to the nucleolar-organizing chromosomes; the distribution at metaphase, at anaphase, and at telophase, with the reorganization of the new nucleolus or nucleoli.

Breakdown of the Nucleolus

There are three chief types of breakdown, with some species showing intermediate types, namely (*a*) gradual dissolution of the nucleolus, (*b*) sudden dispersal of the nucleolus into blobs and granules of material, (*c*) no spectacular breakdown at all, merely a loss of the internal structure and of the nucleolar membrane.

Type (*a*) has been described for *S. crassa* and *S. triformis* (Godward, 1950) in some detail. It is also found in *S. sub-echinata*. Characteristic is the fine state of dispersal to which the material from the nucleolus is brought, so that eventually no actual stained particles can be observed in the nucleus but only a deeper overall staining with iron–alum–acetocarmine (Godward, 1950).

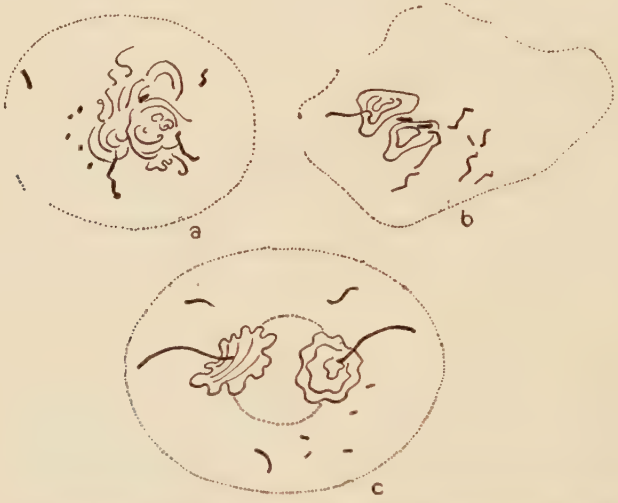
Type (*b*). Figs. 3 and 2, Pl. XXIV, for *S. brittannica* and *S. L.S.* show the explosive breakdown of the nucleolus, the prophase being well advanced. Larger and smaller globules of densely staining material and even splashes of it are scattered about throughout the region occupied by the chromosomes. The nuclear membrane is present still, although there is little sign of it in the photographs. As shown by the figure of Van Wisselingh (Godward, 1950) and of Geitler (1935), a similar stage characterizes *S. setiformis* (seen also by the writer).

Type (*c*). The nucleolus may only lose its organized structure during prophase, viz. the nucleolar tracks disappear, also the nucleolar membrane, but the nucleolar materials are not much dispersed, the region occupied merely undergoes slight expansion. Such appears to be the course followed in Geitler's *Spirogyra X* (1935, p. 12, fig. 1). Yet this may not be the whole story. The stage of explosive dispersal is in the species possessing it, of short

duration since it is not always easy to find. Meanwhile it seems there are several species which like *S. X.* may not show such a stage.

The Distribution of the 'Nucleolar Substance' at Mid-prophase

At mid-prophase a marked redistribution of what at any rate appear to be the materials formerly composing the nucleolus (see discussion) has taken place. This redistribution may be into three portions: (1) the material which in fine or coarse grain is spread throughout the nucleus; (2) the material which as a dense mass or masses, with a vague or clear outline, is still located



Text-fig. 1. *S. brittannica.* Mid-prophase. (*a*) and (*b*) Camera lucida drawings showing the membranes round the N.O. regions of the N.O. chromosomes. (*c*) Reconstructed mid-prophase showing more clearly the relation of the membranes to the N.O. chromosomes.

where the nucleolus was and is apparently in process of being dispersed; and (3) the material which is specially associated with the nucleolar organizing region of the N.O. chromosome. In regard to this last category, we are already faced with the problem that material which is specially associated with a chromosome may be regarded as part of the chromosome, although since it is concerned with dispersing nucleolar material and appears destined itself to be dispersed with it, it may also be regarded as nucleolar substance.

Type (*a*). There are all three of the above portions of nucleolar substance present. This type has been fully described for *S. crassa*, *S. triformis*, and *S. oblata* (Godward, 1950). The nucleolar substance associated with the N.O. chromosome in the first two species forms a finely striated 'lamp-brush'[1] effect round the length of the N.O. regions of the two N.O. chromosomes present; in the last species it consists of granular material forming a hollow cylinder around the N.O. regions. Other examples have now been found. In *S. sub-echinata* (figure to be published in a separate publication) the N.O.

[1] This word is used descriptively; no comparison with the chromosomes of amphibia is intended.

regions are subterminal like those of *S. crassa* and *S. triformis* and the nucleolar substance forms a diffuse cloud around the regions. In *S. brittannica* (Text-fig. 1, *a*, *b*, *c*) the two N.O. chromosomes have each a terminal (or possibly just subterminal) N.O. region; around these regions nucleolar substance is distributed in a very different form from any described hitherto. What appears to be a folded and pleated membrane may entirely surround the region but puffed out from it by intervening non-staining material, like a wrinkled bag. Or the same sort of membrane may form hollow spheres or curved or straight streamers in the neighbourhood of the N.O. region. Another species not yet identified (*S. 4. N.O.*) has four such N.O. chromosomes in the nucleus showing similar membranous matter around the N.O. regions (Text-fig. 2). Here it is mainly present as streamers, and one might say there is a 'lamp-brush' effect on a coarse scale. In both these species a well-defined wrinkled membrane surrounds the organizer track in the nucleolus of the resting nucleus (figure to be published elsewhere), no doubt of the same nature as the membranes observed at mid-prophase.

There can be little doubt that the nucleolar substance thus distributed in various forms around the N.O. regions is in a transitory state, whether it is being removed from the residual mass of nucleolus material, dissolved in the nuclear fluids, or deposited on the chromosomes. Active movement of material is suggested by striations and streamers, an interface between reactants by membranes and by the disposition of granules to form a hollow cylinder. These structures do not stain with Feulgen's stain or methyl green.

Type (*b*). Here there is no special association of nucleolar substance with the N.O. regions, so far as present evidence goes. In *S. setiformis* the N.O. chromosome though thickened is very pale, as are all the other chromosomes of this species, being almost unstained, while dense masses of nucleolar substance are scattered through the nucleus. In other species not identified, where the nucleolar breakdown is apparently of type (*c*), I have observed the N.O. region entering the mass of nucleolar substance, surrounded only by a narrow clear hollow in the heavily stained substance. Possibly no other stages are to be found in these species or they may have been missed. The latter is quite probable.

In Geitler's *Spirogyra X* (1935, p. 12) his text-fig. 1, *a* clearly shows that organizer tracks are present in the resting nucleolus (described as 'unregelmassig geförmte Körper'); N.O. chromosomes are therefore present. His text-fig. 1, *b* shows two chromosomes each having one end attached to the structureless mass of nucleolar substance. These are probably the N.O. chromosomes. No stage showing N.O. regions or specially associated nucleolar substance is figured.

Distribution at Metaphase

The following types of distribution have been observed (Text-fig. 6, *a*, *b*, *c*):

(*a*) No nucleolar substance or only a very diffuse cloud, concentrated round the metaphase plate. Large chromosomes, sticky (Text-fig. 3, and

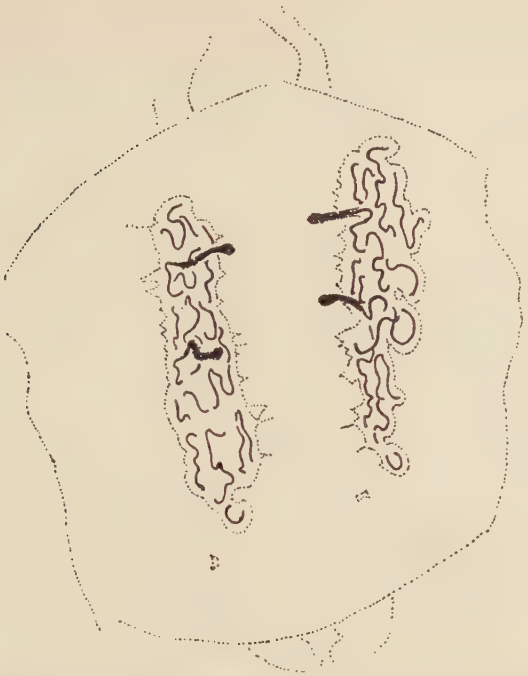
Pl. XXIII, Fig. 1). This type is found in *S. crassa*, *S. triformis*, *S. subechinata*. Of the three species *S. triformis* usually shows the cloud of nucleolar substance, the others less frequently. It is often more concentrated round each chromosome (Pl. XXIII, Fig. 1; and Text-fig. 6, *a*; Text-fig. 3).

TEXT-FIG. 2. Mid-prophase of *S. 4.N.O.* (see photograph, Pl. XXIV, Fig. 1). The drawing shows all 4 N.O. chromosomes each with a 'lamp-brush' region; only 2 were in focus in the photograph. Two globular masses of N.S. are not yet dispersed.

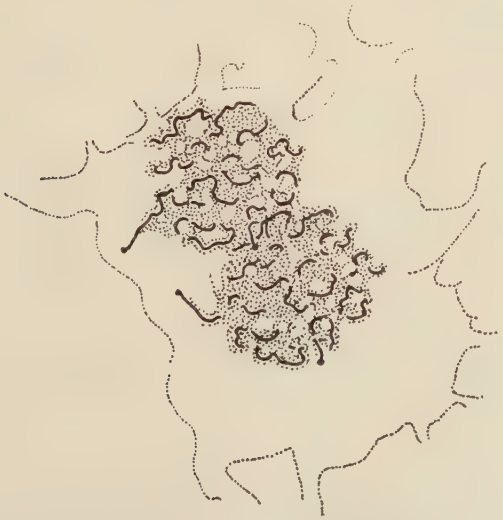
TEXT-FIG. 3. Sticky metaphase chromosomes of *S. triformis*.

(*b*) Metaphase chromosomes embedded in a plate of structureless nucleolar substance which, however, stains less heavily than the chromosomes and is relatively transparent (Text-fig. 6, *b*). This is found in *S. submargaritata* and two species as yet unidentified (*S. 4.N.O.* and *M.1*). The chromosomes are of medium size and are not sticky.

(*c*) Metaphase chromosomes embedded in a dense mass of granular nucleolar substance. This is striated in the same sense as the spindle-fibres. The chromosomes are small or minute or (*S. setiformis*) unstainable. They are not sticky (Text-fig. 6, *c*, *d*; Pl. XXIII, Fig. 4). In this type the nucleolar substance most resembles, in its degree of concentration and depth of staining, the nucleolus itself, and confusion with the nucleolus

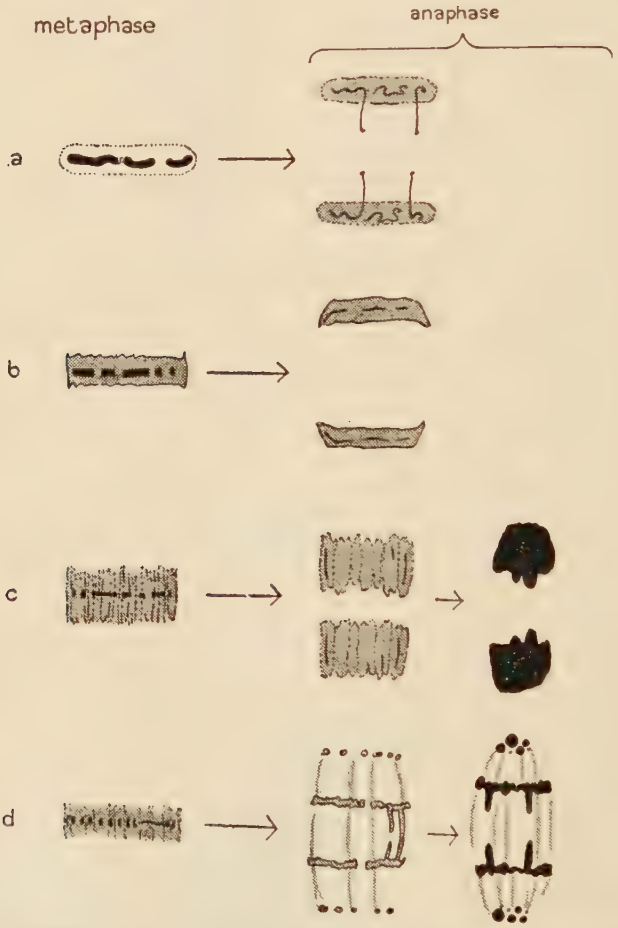


TEXT-FIG. 4. Anaphase, *S. crassa* (cf. Text-fig. 6, *a*). N.S. is seen being sloughed off the chromatids; N.O. regions hang behind the chromatid plates.



TEXT-FIG. 5. *S. crassa*, anaphase, flattened, showing the nucleolar substance sloughed off the chromatids and forming a coarse network connecting them.

could most easily arise in observing these species. It is easily distinguished from a nucleolus, however, in being devoid of organizer track structure, shaped like a flat thick plate instead of spherical, composed of granular material which is arranged to fit between the spindle-fibres and seems perhaps to have become part of the spindle.



TEXT-FIG. 6. Relations of N.S. and chromosomes at metaphase and anaphase. Types (*a*), (*b*), (*c*) of metaphase and (*a*), (*b*), (*c*), (*d*) of anaphase. Earlier and later anaphase shown for (*c*) and (*d*). (*a*) e.g. *S. crassa*; (*b*) e.g. *S. 4.N.O.*; (*c*) e.g. *S. brittannica, S. setiformis*; (*d*) e.g. *S. oblata.*

This type of distribution is the commonest and many species as yet unidentified have been found to have it besides the following: *S. daedalea, S. maxima, S. gracilis, S. oblata, S. setiformis*, and unidentified *S. L.S.* and *S. F.* Type (*c*) was observed by Geitler in *S. setiformis* and *S. X.* (1935).

Grouped in the sequence adopted above, the species illustrate a progressive increase in the amount of nucleolar substance visible round the metaphase

plate. It should be noted that in most of them there is even at metaphase a general diffusion through the nucleus as well as a concentration round the chromosomes. The only species which do not have much, if any, nucleolar substance at the equator of the spindle are those of type (*a*), which are also the only species having large deeply staining chromosomes with a tendency to stickiness at metaphase. It would be reasonable to assume that the deep staining and stickiness of these chromosomes are due to the deposition on them of the nucleolar substance which is not visible (except as a pale diffusion) elsewhere in the nucleus. In the species of type (*c*), at the other extreme, the chromosomes being small, for this reason or another the nucleolar substance is not deposited on them; instead it remains free in the nucleus. Most of it tends to concentrate at the equator of the spindle surrounding the chromosomes.

Distribution at Anaphase

Before describing the peculiarities of the distribution of the nucleolar substance at anaphase in the different species it is as well to recall by what right the substances referred to are still called 'nucleolar substance'. We have seen the nucleolus broken down into materials which may arrange themselves on the metaphase plate with the chromosomes or possibly deposited on the surface of the metaphase chromosomes. These are the materials whose distribution in anaphase is now followed, the name 'nucleolar substance' still being used for them.

At anaphase the following types of distribution occur (Text-fig. 6, *a*, *b*, *c*, *d*):

Type (*a*). (Here the chromatids lie parallel to each other and to the equator: this is a question of centromere organization and will be dealt with elsewhere.) The nucleolar substance which, it has been suggested, formed a sticky coating to the chromosomes at metaphase, is progressively sloughed off the chromatids during the course of anaphase, ultimately forming a complete or partially complete network of material in which the chromatids, now somewhat attenuated, are embedded (Text-fig. 4 and 5, 6, *a*; Pl. XXIII, Fig. 2). The N.O. regions characteristically hang back from the chromatid plate but are also enveloped in nucleolar substance. Such is the state of anaphase in *S. crassa*, *S. triformis*, and *S. sub-echinata*.

Type (*b*). (The chromatids lie parallel or not, often they are small.) The plate of nucleolar substance already present at metaphase divides when the chromosomes do into two flat plates of nucleolar substance containing the chromatids, which pass to the poles (Text-fig. 6, *b*). This is a common type; the chromatid plates often are not flat when the chromatids themselves are aligned with the spindle-fibres instead of the equator, or when they are very small.

(*b* 1) (with flat plate): *S. sub-margaritata*, *S. 4.N.O.*, *S. M.1.*, *S. X.* (Geitler, 1935).

(*b* 2) (thicker more elongate chromatid plate): *S. gracilis*, *S. maxima*, *S. L.S.*, and many others.

It is fairly common for the N.O. chromatids or regions to project towards the equator from the anaphase mass (as in type (*a*), for example in *S. setiformis* (Text-fig. 6, *c*).

Type (*c*). The nucleolar substance becomes progressively more closely associated with the chromatids as anaphase proceeds—one might say it becomes deposited on them (cf. Geitler, 1935). Each chromatid (orientated parallel with the spindle-fibres) can be seen in an attenuated condition within a coating of nucleolar substance whose outline, however, is far from well defined at mid-anaphase (Text-fig. 6, *c*). Not till late anaphase does the reverse process of sloughing-off set in leading to the formation of a mass of nucleolar substance in which the chromatids—what little can be seen of them —are embedded (Text-fig. 6, *c*). Examples of this type are *S. brittannica*, *S. F.*, *S. setiformis*.

S. setiformis is the species for which the process of deposition of nucleolar substance on the anaphase chromatids was first described by Geitler (1935). My interpretation of the anaphase of this species, however, while agreeing with his on this main point, disagrees in other respects. His figures (1935, fig. 3, p. 15, *e* and *f*) would lead one to suppose that the chromosomes were themselves becoming more clearly visible by this deposition of nucleolar substance and that at the end of anaphase (fig. *f*) each chromatid was a thick clearly defined object already showing the longitudinal split in preparation for the next division! That this is not the case can most clearly be seen by comparing *S. setiformis* with *S. brittannica* or *S. F.* (Text-fig. 6, *c*; Pl. XXIII, Fig. 3). In these species the chromosomes at metaphase stain reasonably, in contrast to those of *S. setiformis*. At mid-anaphase the chromatids of the two former species are clearly visible, and each is no more than the thinnest chromonema enclosed in its sheath of nucleolar substance, which in some instances may be continuous with that of the next adjacent chromatid. This nucleolar substance which was striated at metaphase in a sense parallel to the spindle-fibres is still to some extent striated in this way. At late anaphase the whole of the nucleolar substance has become fused to a single mass which still shows striations but in which the chromatids, presumably as a result of further attenuation, are no longer visible. Projecting from the anaphase mass towards the equator may be the two 'horns' of nucleolar substance (Text-fig. 6, *c*) which accumulated round the N.O. regions of the N.O. chromosomes—in these also at this stage no chromonema is visible. The same stages, so far as the nucleolar substance is concerned, are found in *S. setiformis*, the difference being that the chromosomes are almost unstained at metaphase, so that they are uncountable; and no sign whatever of the actual chromatids can be seen in anaphase; all that is seen is the nucleolar substance which is being concentrated round them. The projecting N.O. regions are indicated as in the other species. The nucleolar substance is striated in all species; it is this striation, I believe, which has been drawn, together with some indication of the projecting 'horns', in Geitler's figure (1935, p. 15, *f*).

Type (*d*). Only one species so far comes into this category; it is *S. oblata* (formerly tentatively identified as *?ellipsospora* by the present writer (1950), but now that zygospores have been observed it is certainly known to be *oblata*). The chromatids are minute and there is some but not a dense mass of nucleolar substance concentrated round the metaphase plate. It is striated in the same sense as the spindle-fibres. The chromatids are distinct. As they pass towards the poles, however, the deposition of nucleolar substance on and around them begins and continues till the chromatid plate is covered with an irregular mass of nucleolar substance, the N.O. regions projecting from the mass in the direction of the equator (Text-fig. 6, *d*; Pl. XXIII, Fig. 5). Nucleolar substance is also seen in long fibrils, being drawn out in the spindle; blobs of it at first small, later larger and more numerous, appear at the poles in advance of the chromatid mass. Clearly a part of the nucleolar substance here has been moved by the spindle, but more quickly than the chromatid plate. Some might be inclined to say that it was part of the structure of the spindle. The sloughing-off of the newly deposited nucleolar substance from the chromosomes apparently supervenes as the end of anaphase is reached. Nothing is seen of the chromatids themselves from early anaphase onwards.

In the various species at anaphase we can see four processes through which the nucleolar substance may pass: (1) continuous contact with chromatids, maintained from metaphase through anaphase; (2) deposition on the chromatids occurring during anaphase; (3) sloughing-off the chromatids, occurring towards the end of anaphase; (4) movement to the poles independent of the chromatids. The striation of the nucleolar substance seen often at metaphase and during part of anaphase, as well as the movement of it in the spindle in one species independently of the chromatids, and in the others with the chromatids, imply a close connexion between the substance and the functioning of the spindle.

Distribution at Telophase

Whatever the means which may have been adopted during anaphase to achieve it, the distribution of nucleolar substance at the beginning of telophase is the same in all species. There is the 'chromatid plate' whose chromatids are frequently already so attenuated as to be invisible to the eye, embedded in a dense mass of nucleolar substance of variable shape, sometimes a uniform plate, sometimes irregular and perforated or separated into blobs. Very soon this substance begins to dissolve, and if blobs were not present before they now appear, usually spherical, large and small. As the mass breaks up and the blobs become paler, the N.O. regions begin their activity and new nucleoli begin to be organized, very small at first, equal in number of course to the number of N.O. chromosomes present, and soon showing the characteristic organizer track structure of the particular species. At the time when the N.O. regions first begin their activity they are visible only as a consequence of it. As the nucleoli increase in size they may fuse. Meanwhile the nucleolar substance which has been dissolving may be used up completely or one, two,

or more small spheres of it may remain to be incorporated as they are in the resting nucleus.

It would appear from the course of telophase that whatever the anaphase origin of the nucleolar substance with which telophase begins, it is used up as the nucleolus proper is organized, presumably in the formation of the nucleolus.

Discussion

The following questions are perhaps suggested by the foregoing study.

1. Is the substance which has been referred to as nucleolar substance directly derived from the nucleolus during its breakdown? So far as I can see there is no evidence for or against this. If it is not directly, then it is indirectly derived from the nucleolus as a result of chemical changes of which nothing is known for certain, except (as would be expected) that there is no desoxyribose nucleic acid present.

2. Is it reasonable to use the term 'nucleolar substance' at all, once the nucleolus has definitely ceased to exist as such, for materials which are seen in such different forms during mitosis? It may seem to be giving a false impression to use it. However, it seems better to follow the example set by Geitler until more is known of the cytochemistry, when the term may become unnecessary, than to invent new terms. Nevertheless it may be that had Geitler known of the existence and activities of the N.O. chromosomes and of the extent of the difference between the organized nucleolus and the 'nucleolar substance', he might not have adopted the term.

3. Is the genus *Spirogyra* a mere freak of passing interest in regard to the behaviour of the nucleolar substance, or has this behaviour any significance for the general interpretation of mitosis? It is a textbook commonplace that the nucleolus breaks down, its ribosenucleic acid is converted to desoxyribose nucleic acid in the act of being deposited on the chromosomes, and this is the cause of the contraction and deep staining of the chromosomes towards metaphase. Then at telophase the desoxyribose nucleic acid comes off again and a new nucleolus appears composed of derived ribosenucleic acid. Yet in most (all?) nuclei, all that can be seen to bear out the story is the appearance and disappearance of the nucleolus, with the concomitant disappearance and appearance of the stainability of the chromosomes. Only in *Spirogyra* does the breakdown of the nucleolus give a visible substance which can be seen in the act of depositing itself on the chromosomes and coming off them again, then dissolving up as the nucleolus is formed, as required by the theory. The nucleic acid cycle perhaps moves slowly; certainly it moves rather differently in the different species. For example, in *S. crassa* deposition on the chromosomes is almost complete at metaphase; in *S. setiformis* it hardly occurs at all; the other species come between the two, giving an interesting range, of time, manner of, and degree of deposition. Other points involved in this issue will be raised elsewhere.

4. Can Geitler's main conclusion, based on three species (given in trans-

lation on p. 404), be unreservedly supported? Not perhaps unreservedly, although to a large extent it can be. For example, that the closeness of the relationship of the nucleolar substance to the chromosomes varies; that the loading of chromosomes with the substance during anaphase can be followed. Not, however, that in *S. crassa* the substance seems permanently attached to the chromosomes (see anaphase, *S. crassa*), not entirely that the nucleolar substance may appear as chromosome substance. Certainly it seems like it in many cases and especially in the type of *S. crassa*. When one considers the meaning of 'chromosome substance', however, one is faced in *Spirogyra* in, for example, the metaphase chromosome, with the deeply staining chromosome material as ordinarily understood and with outer coatings and coverings of variable extent and definiteness. In *S. crassa* the outer coating causing stickiness has been referred to elsewhere (Godward, 1950*a*) as a 'fluid matrix'.

It seems, from a comparison of the chromosomes of all species at metaphase, that the amount of nucleolar substance forming an outer investment is inversely proportional to the size and depth of staining of the chromosome. Therefore perhaps it is to be inferred that the nucleolar substance first forms an outer covering of diffused material, which gradually condenses on the chromosome, thus becoming 'chromosome substance'. This has been more or less assumed in the previous paragraph (3). It would seem that this condensation is seldom carried far; that it is only near completion in the three species of the *crassa* type; and that the reverse process of sloughing-off setting in during anaphase has the result that in very few *Spirogyra* species are the anaphase chromatids more than just visible if at all, although their position is indicated by the associated nucleolar substance.

Acknowledgement

I am indebted to Professor I. Manton and Mr. Brian Clarke for Pl. XXIII, Fig. 1.

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DESCRIPTION OF PLATES

Illustrating M. B. Godward's article on 'Geitler's Nucleolar Substance in *Spirogyra*'.

PLATE XXIII

Metaphase-anaphase distribution of nucleolar substance.

Fig. 1. Metaphase, *S. sub-echinata*, type (*a*). Diffuse N.S. around chromosomes. (×4,000.)

Fig. 2. Anaphase, *S. sub-echinata*, type (*a*). N.S. being sloughed-off chromatids forms a marked investment. Chromatids are visible. (×4,000.)

Fig. 3. *S. brittannica*, anaphase, type (*c*). No chromatids are seen, but their place is marked by the N.S. covering deposit. (×1,500.)

Fig. 4. *S. oblata*, metaphase, type (*c*). Small chromosomes, striated N.S. at equator. (×2,000.)

Fig. 5. *S. oblata*, anaphase, showing deposit of N.S. on chromatids rendering them invisible, and globules of N.S. which have reached the poles of the spindle before the chromatids. Note the N.O. regions of the N.O. chromosomes hanging back to the equator (marked by N.S. covering deposit). (×2,000.)

PLATE XXIV

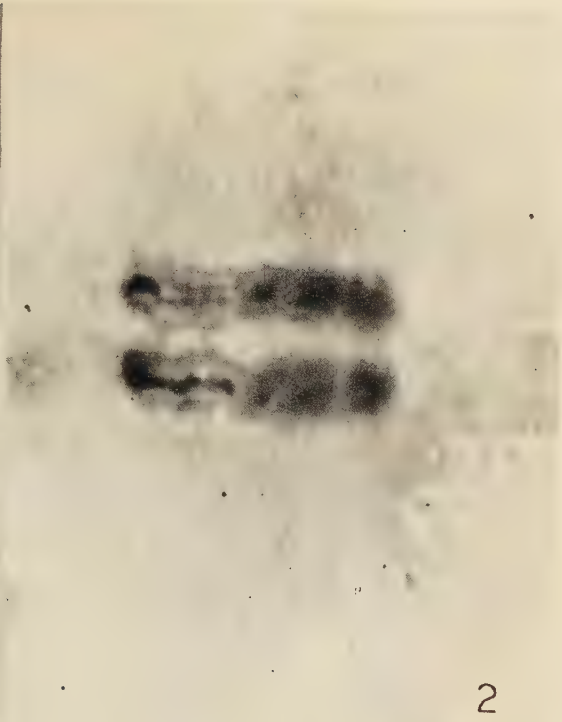
Prophase distribution of nucleolar substance.

Fig. 1. *S. 4.N.O.* (cf. Text-fig. 2); two of the four N.O. ('lamp-brush') chromosomes are in focus, with two blobs of nucleolar material not yet dispersed. (×5,000.)

Figs. 2 and 3. Sudden ('splash') dispersal of N.S. from nucleolus. *S. L.S.* and *S. brittannica*. Prophase chromosomes are visible. (×1,100 and ×3,000.)

N.S. = nucleolar substance.
N.O. = nucleolar organizing.


1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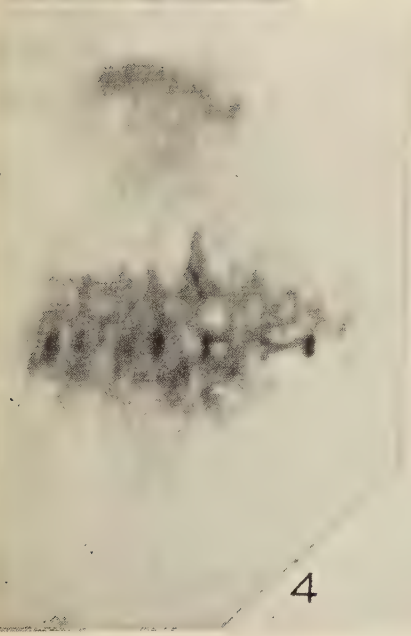
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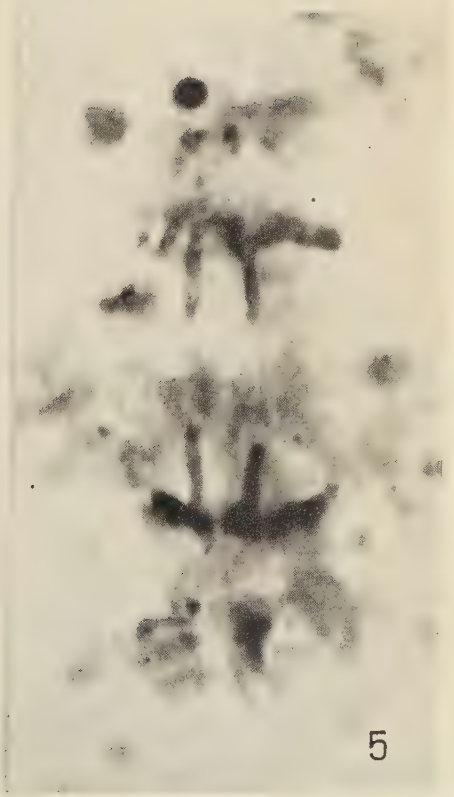
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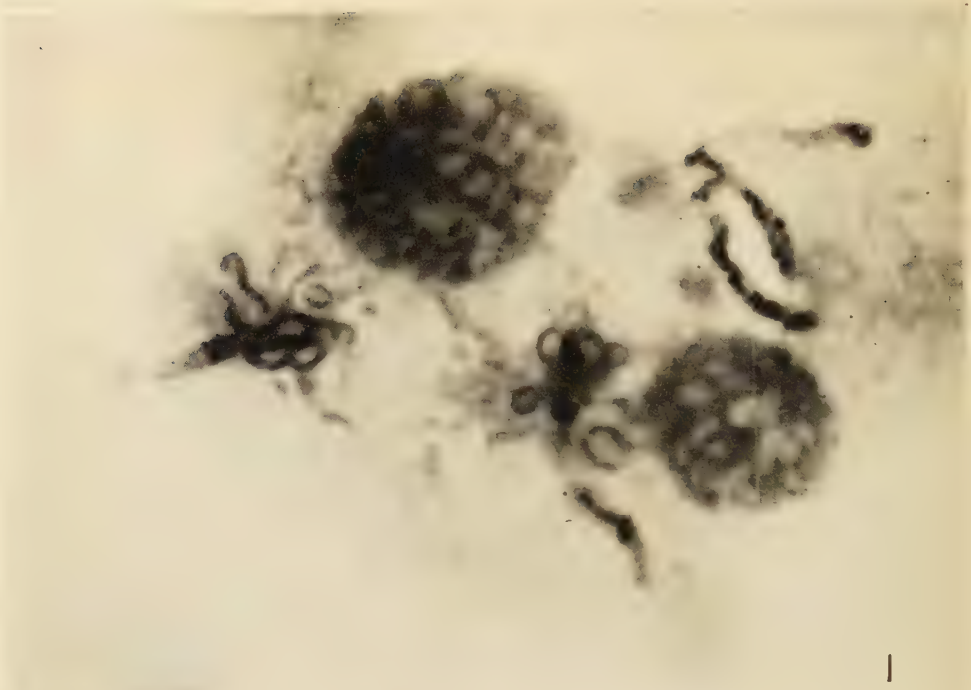

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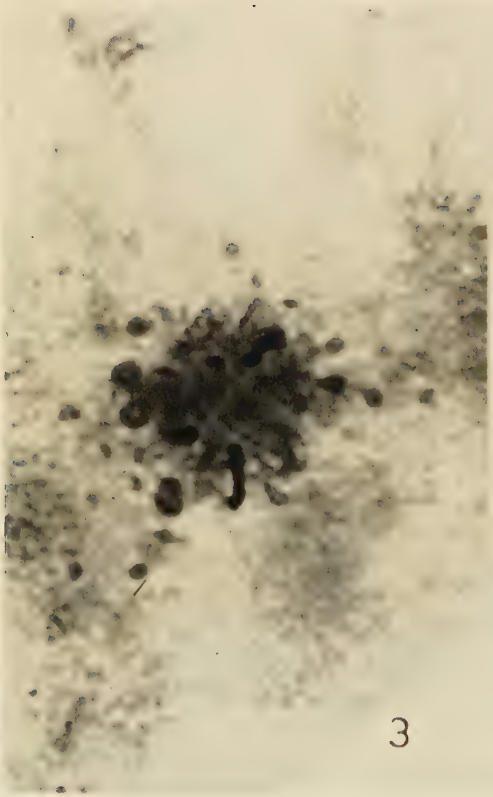

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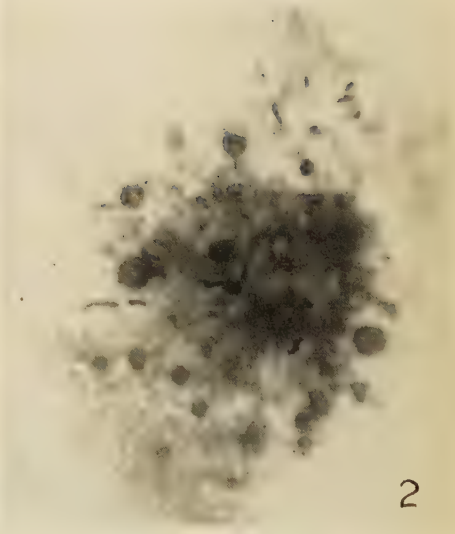

2

Vernalisation of Winter Rye by Negative Temperatures and the Influence of Vernalisation upon the Lamina Length of the First and Second Leaf in Winter Rye, Spring Barley, and Winter Barley

BY

H. HÄNSEL

With four Figures in the Text

Abstract

The lowest effective temperature observed for vernalising Petkus Winter Rye was $-4{\cdot}5^{\circ}$ C., whereas -6° to -10° C. did not induce any hastening of flowering. Seedlings of 20–25 mm. length were as susceptible to -3° C. as seeds in the stage at which the radicle had just broken through the pericarp. Vernalising temperatures alternating between 7 days $+1^{\circ}$ C. and 3 days -3° C. were as effective as continuous treatment by $+1^{\circ}$ C. The lamina length of the first and second leaf is shortened by raising the vernalising temperatures from -3° C. to $+3^{\circ}$ C. and also is further shortened by prolonging the vernalisation period in winter rye and winter barley. No progressive shortening of the lamina length, however, was observed in spring barley when treated like winter barley. This suggests a direct dependence of the lamina length of the first and second leaf on the degree of vernalisation reached in the embryo.

[Annals of Botany, N.S. Vol. XVII, No. 67, July, 1953.]

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Introduction

GASSNER (1918) exposed germinated seeds of winter rye for different periods to temperatures from −5° C. to 0° C. with the result that a 25 days' exposure already showed a vernalising effect. As the temperature of the cold mixture used increased gradually from −5° C. to 0° C. no conclusion could be drawn about the lowest vernalising temperature effective in this experiment. Experiments carried out with winter barley (Hänsel, 1949) gave evidence that plants sown in the autumn reached a higher degree of vernalisation after a 3 weeks' period of, on the average, −4·1° C. Germinated seeds of the same varieties were exposed to the following course of temperature: 15 days at −5° C. to −4° C., 5 days at −2° C. to −1° C., and 10 days at about 0° C. This treatment resulted in a very similar effect to a continuous exposure to about 0° C. for 30 days, but the effect of definite negative temperatures could not be isolated as the refrigerator could not be kept at constant negative temperatures. In another experiment (Hänsel, 1949, unpublished) several varieties of winter barley were vernalised by constant temperatures of −2° C. and −4° C. However, the small percentage of surviving plants did not show a definite effect of the preceding cold treatment, which might have been due to the temperatures during early growth often exceeding 25° C., since Purvis and Gregory (1952) found that partially vernalised grains are devernalised more easily than completely vernalised grains, and Purvis (1948) mentioned that even temperatures of +18° C. could induce some devernalisation.

The following experiments attempt to determine the vernalising effect of definite negative temperatures and the lower limit for the vernalisation of winter rye and extend the results at low positive temperatures already obtained by Purvis (1948), thereby completing the action curve on the negative side of 0° C. After having completed the experiments it was learnt that Razumov, Feofanova, and Oleinkova (1948) obtained a vernalising effect by treating germinated grains of winter wheat and winter rye at −3·8° C. and −6° C. A vernalising effect of −6° C. could not be confirmed in the experiments presented here. Vernalisation by alternating temperatures should elucidate in how far the rate of vernalisation could be changed by shorter and longer periods of intermitting negative temperatures. The question whether there is an influence of vernalisation on the subsequent growth of first and second leaf (Thimann and Lane, 1938; Denffer, 1939; and Konovalov, 1944) was pursued by measuring lamina length of first and second leaf after different cold treatments of winter rye, spring barley, and winter barley.

Vernalisation of Petkus Winter Rye by Temperatures ranging from +3° C. to −10° C.

Two experiments were carried out, one at temperatures from +3° C. to −3° C. and subsequent growth in a warm house (winter 1949/50), the other

at temperatures from +1° C. to −10° C. and subsequent growth in the open air (spring 1950).

1. *Technique of vernalisation*

Seeds of Petkus winter rye, strain RN 19, provided by the National Institute for Agricultural Botany, Cambridge, were first disinfected with 0·1 per cent. sublimate saponin solution, washed, and submerged for 12 hours in distilled water. After pouring off the water they were kept at room temperature till 50 per cent. of the grain showed roots broken through the pericarp. In this stage the seeds were placed in Petri dishes, 50 in each, on a single layer of wet blotting-paper, and transferred to different cool chambers. Seeds which had to be treated by negative temperatures were hardened gradually, for 2 days to −3° C. in the first, and for 3 days to −10° C. in the second experiment respectively. The same time was employed for the procedure of thawing at the end of the chilling treatment. To increase the moisture content of the air in the Petri dishes without promoting growth of the grain by direct water-supply, about every fortnight, one blotting-paper, wet with water at +1° C., was inserted on the inside of the covering dish. This was done uniformly for all treatments. The growth of the embryo was almost completely suppressed by temperatures of −2° C. and below, as seen in Table I.

TABLE I

Length of the Coleoptile in mm. of Petkus Winter Rye after Vernalisation by Different Temperatures

	Duration of vernalisation in days.	Temperature of vernalisation, °C.								
		+3	+1	0	−0·6	−2	−3	−4·5	−6	−10
1st experiment	45	20	15	8	7	1	1	—	—	—
2nd experiment	67	—	20	12	—	—	1	1	1	1

The fluctuations of the different temperatures during the cold treatment were reasonably small (Table II) except in the −6° C. chamber, where a daily fluctuation between −5° C. and −7° C. had been aimed at. From the 12th (first experiment) and 9th (second experiment) day onwards, seeds treated with temperatures below −0·6° C. were covered with ice crystals.

TABLE II

Absolute Minima and Maxima in the Cold Chambers during Vernalisation

	Duration of vernalisation in days.		Temperature of vernalisation, °C.								
			+3	+1	0	−0.6	−2	−3	−4·5	−6	−10
1st experiment	45	Min.	3·0	0·8	0·2	−0·6	−2·8	−3·0	—	—	—
		Max.	3·8	1·3	0·0	−0·2	−1·5	−2·5	—	—	—
2nd experiment	67	Min.	—	1·0	0·2	—	—	−3·3	−4·8	−8·0	−11·8
		Max.	—	1·3	0·4	—	—	−2·8	−4·2	−5·2	−9·2

To determine the rise in temperature produced by respiration during the cold treatment the temperature in a 2-cm. layer of germinated seeds in a conical flask closed by cotton-wool was measured during the first 3 weeks in the +1° C., −3° C., and −6° C. chambers. In the +1° C. chamber the temperature within the seed bulk was found to increase by +0·5° C. to +0·8° C. during the first 4 days. The seed bulk stored in the −3° C. chamber showed slightly lower temperatures than the ambient, the differences ranging from −0·2° C. to −0·8° C. The temperatures in the −6° C. chamber fluctuated too widely to allow of conclusive results. The combined effects of heat production due to respiration and cooling due to evaporation may be responsible for these differences. Since the temperature changes in a bulk of seed were so small it may be assumed that in widely spaced grains as used in the Petri dishes the effects would be negligible.

2. *Planting*

The vernalised seeds and the germinated control seeds were planted out in earthenware pots, 7 in each and later reduced to 6 per pot. The pots were randomized in blocks with 5 in the first and 4 replications in the second experiment. The natural short day during December and January (first experiment) was supplemented to a 17-hour day by 40-W. Osram bulbs, producing about 400 lux at pot level. The second experiment was planted out on April 25, the natural day length being 14½ hours and increasing to 15 hours within 10 days. In both experiments the temperatures during early growth ranging from +10° C. to +18° C. were low enough to prevent devernalisation almost completely, although a slight amount of natural vernalisation might have occurred during growth by temperatures about +10° C. As all treatments, including the control, were under the influence of these temperatures, no essential alteration of the relative results was to be expected.

3. *Presentation of results*

'Shoot height', i.e. the distance between the base of the spike and of the culm, and 'scores' evaluating the stage of the differentiation of the spike, were used to estimate the amount of progress towards flowering at the time of dissection. The conventional 'scores' scale (Gregory and Purvis, 1938) with 49 units up to anthesis was enlarged by 5 units between the stages 35 and 40. Consequently 10 units were provided for stages between 'awns growing' and 'spike emerged from last leaf'. The corresponding morphological stages are:

35 awns growing
36 awns cover flower
37 awns meet over top of the spike
38 awns 1–2 cm. above top . . . .
39 top degenerates
40 spikelets spreading
41 awns emerging from last leaf
43 basis of spike visible
45 spike fully emerged

54 anthesis

A rapid elongation of spike and culm accompanied these stages of differentiation.

Purvis (1948) stated that 'the frequency distribution of "scores" in a sample of partially vernalised plants is definitely asymmetrical', which is in agreement with the present observations in lots of plants vernalised by temperatures of −2° C., −3° C., and −4·5° C. Nevertheless, 'scores' were treated statistically in the usual way for the estimate of variance. No analysis of variance was applied to 'scores' of winter rye as the variance in the different temperature treatments proved to be significantly different ($P = 0{\cdot}05$). The same applies to the variance of shooting data in rye. In lamina length the variance proved to be independent of the mean, however, only within each group of first and second leaves, so that the minimum significant difference (m.s.d.) for the 0·05 and 0·01 level was calculated separately for the first and second lamina. In the last experiment carried out with barley, the variance of 'scores' showed no dependence on the mean, thus making possible an unbiased estimate of a minimum significant difference. The more homogeneous genetical material of barley due to self-fertilization may be responsible for this fact.

4. *Results*

(*a*) *The lower limit of vernalising temperatures.* Combining the results of 'scores' and 'shoot height' of both experiments as shown in Table III and Fig. 1, it is seen that negative temperatures down to −4·5° C. had a vernalising effect.

The differences between each of the treatments between −0·6° C. and −6° C. are well established and prove a gradually decreasing vernalising effect. In the second experiment one control (control 'hardened' in Table III) was hardened to −6° C. and subsequently thawed with the −6° C. and −10° C. series before planting, and as was to be expected, hardening and thawing temperatures (during 6 days respectively) did not enhance subsequent spike differentiation.

One pot of each series of the second experiment was left for further observation. The data for flowering and spike emergence of these plants, given in Table IV, show clearly the effectiveness of temperatures down to −4·5° C., and leave little doubt that the treatment with −6° C. and −10° C. neither hastened nor delayed shooting when compared with the control plants. The null point of vernalisation in this experiment lay somewhere between −4·5° C. and −6° C.

(*b*) *Influence of the stage of growth.* To investigate whether seedlings in a more advanced stage of growth responded differently to negative temperatures, seedlings 20 to 25 mm. long were vernalised by negative temperatures simultaneously with the normal series. On the fourth day all treatments except those stored in the −0·6° C. chamber were completely frozen. All seedlings of the −6° C. and −10° C. decayed after planting. Comparing the results of the advanced stage of growth (Table V) with those of the normal sets, no hastening of spike differentiation due to the more advanced stage of

Table III

'Scores' and 'Shoot Height' of Petkus Winter Rye vernalised by Temperatures ranging from +3° C. to −10° C. (Replicate Numbers in Brackets)

Temperature of vernalisation, °C.	1st experiment. Duration of vernalisation: 45 days. Date of planting: Dec. 6, 1949. Time from planting till dissection: 49 days. Grown in warmhouse under 17-hr. day		2nd experiment. 67 days. April 25, 1950. 48 days. Grown in open air under natural day length	
	'Scores.'	'Shoot height' mm.	'Scores.'	'Shoot height' mm.
+3	(24) 37** ± 0·4	(28) 169** ± 10·4	—	—
+1	(22) 35 ± 0·2	(23) 115 ± 7·7	(15) 45 ± 0·6	(15) 303 ± 18·4
0	(22) 35* ± 0·4	(22) 123 ± 10·5	(23) 44** ± 0·3	(23) 302** ± 18·4
−0·6	(28) 34** ± 0·4	(28) 108** ± 7·9	—	—
−2	(21) 29** ± 0·7	(21) 51** ± 11·5	—	—
−3	(30) 26** ± 0·6	(30) 19** ± 3·8	(21) 40** ± 0·4	(21) 144** ± 10·2
−4·5	—	—	(17) 27** ± 0·5	(17) 26** ± 3·8
−6	—	—	(16) 15 ± 0·3	(16) no shooting
−10	—	—	(14) 14 ± 0·4	(14) ,, ,,
Control germinated at 18° C.	(27) 15 ± 0·2	(27) no shooting	(19) 15 ± 0·3	(19) ,, ,,
Control 'hardened'	—	—	(21) 15 ± 0·2	(21) ,, ,,

Means marked with a single and double asterisk respectively are different from all means below at least at a 5% and 1% level respectively.

growth can be detected in the −3° C. series. The delay due to the more advanced stage in the −0·6° C. series when compared with the normal series treated at 0° C. may be due to the different temperatures. This observation is on the same lines as those made by Purvis (1944), who found that the different growth of embryos during vernalisation due to different sugar content of the nutritive medium showed no correlation with the final degree

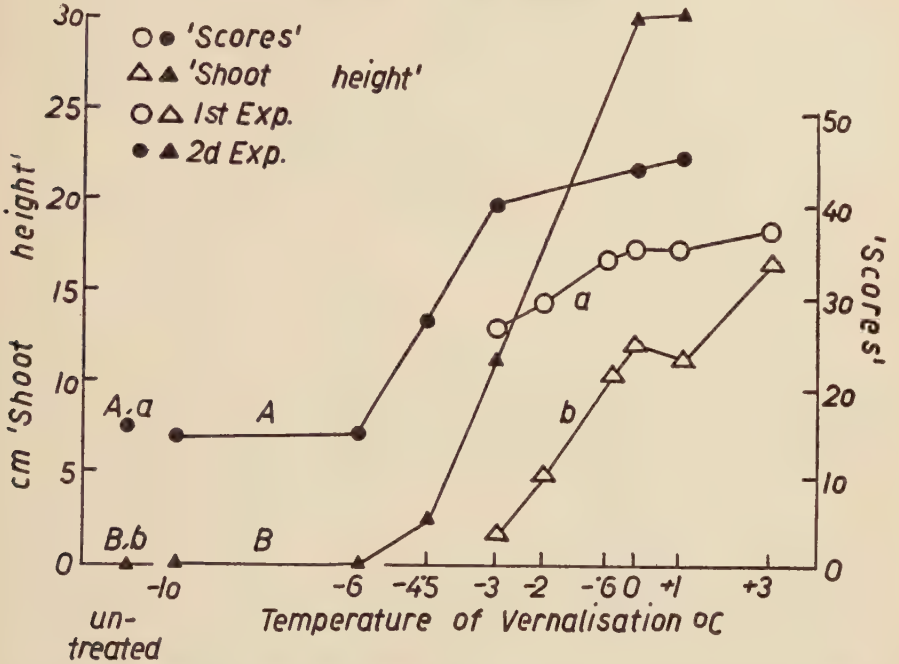

FIG. 1. The effect of vernalising temperatures from +3° C. to −10° C. on subsequent progress to flowering as shown by 'shoot height' (B,b) and 'scores' (A,a) when grown under long days. Duration of vernalisation: 1st expt. (a, b) 45 days, 2nd expt. (A, B) 67 days. Dissection: 1st expt. 49 days, 2nd expt. 48 days after planting.

of vernalisation. The independence of growth rate and stage of growth of the seedlings and the vernalising effect is one of the conditions of an unbiased comparison of the vernalising effect of different temperatures.

(*c*) *The vernalising effect of temperatures alternating between positive and negative degrees Centigrade.* In the first experiment one series was transferred every fifth day from +1° C. to −0·6° C. and vice versa. Three series of alternating temperatures were tested in the second experiment:

Treatment A: 3 days +1° C., alternating with 7 days −3° C.
,, B: 5 days +1° C., alternating with 5 days −3° C.
,, C: 7 days +1° C., alternating with 3 days −3° C.

The results of these treatments compared with the corresponding constant temperature treatments are given in Table VI. In the first experiment only the 'shoot height' of the treatment alternating between +1° C. and −0·6° C.

TABLE IV

Time of Flowering and Emergence of Spike respectively (Single Pot of 2nd Experiment)

Temperature of vernalisation, °C.	Number of plants.	Number of flowering plants.	Time of flowering.
+1	6	6	June 21 to 26
0	6	6	June 20 to 26
−3	6	6	June 26 to July 2
−4·5	6	5	June 30 to July 8
		Number of plants shooting before Sept. 15.	Date of spike emergence.
−6	6	0	Aug. 26
−10	5	2	Aug. 28, Sept. 1
Control germinated at 18° C.	6	1	Aug. 25
Control 'hardened'	6	2	Sept. 3, Sept. 11

TABLE V

Influence of Different Stage of Growth on the Susceptibility for Vernalising Temperatures in Petkus Winter Rye. (Duration of Vernalisation 67 Days, Plants dissected after 48 Days of Growth. Replicate Numbers in Brackets)

Temperature of vernalisation, °C.	Coleoptile 20–25 mm. long when placed in cold chamber		Temperature of vernalisation, °C.	50% radicles broken through pericarp, when placed in cold chamber	
	'Scores.'	'Shoot height' mm.		'Scores.'	'Shoot height' mm.
−0·6	(21) 42 ± 0·6	(21) 252 ± 14·7	0	(28) 44 ± 0·3	(23) 302 ± 18·5
−3	(22) 40 ± 0·4	(22) 114 ± 11·1	−3	(21) 40 ± 0·4	(21) 114 ± 10·2

exceeded clearly those of the constant −0·6° C. treatment. In the second experiment only the 'shoot height' of alternating treatment 'A' with 3 days' −3° C. intervals exceeded the continuous +1° C. treatment, but the 'scores' did not show a significant difference. Considering the values of 'scores' only, it is evident that the 3 days' periods of −3° C. in treatment 'A' did not delay the process of vernalisation when compared with a continuous +1° C. treatment under the circumstances described.

Considering the results of treatment 'B', alternating between 5 days +1° C. and 5 days −3° C., it can be stated that 'scores' and 'shoot height' are much closer to the continuous +1° C. than to the continuous −3° C., though obviously 50 per cent. of the duration of treatment 'B' occurred at each of these temperatures. Taking difference between the two continuous treatments as 100 per cent., treatment 'B' reaches 80 and 86 per cent. of the difference in 'scores' and 'shoot height' respectively. To account for this fact, as well as for the equal rate of vernalisation of the treatment 'A' (about $\frac{1}{3}$ of the time at −3° C.) and the continuous treatment at +1° C., three suggestions

Table VI

Vernalising Effect of Temperatures alternating between Negative and Positive Degrees C. on Petkus Winter Rye (Replicate Numbers in Brackets)

Temperature of vernalisation °C.	1st experiment. Duration of vernalisation, 45 days. 'Scores.'	1st experiment. Duration of vernalisation, 45 days. 'Shoot height' (mm.)	Temperature of vernalisation, °C.	2nd experiment. Duration of vernalisation, 67 days. 'Scores.'	2nd experiment. Duration of vernalisation, 67 days. 'Shoot height' (mm.)
+1	(22) 35* ± 0·2	(23) 115** ± 7·7	+1	(15) 45 ± 0·6	(15) 303** ± 18·4
Alternating R	(25) 36** ± 0·4	(25) 115** ± 8·4	Alternating A	(14) 45 ± 0·9	(24) 350** ± 17·2
−0·6	(28) 34 ± 0·4	(25) 108 ± 7·9	Alternating B	(22) 44** ± 0·5	(22) 276* ± 12·4
			Alternating C	(24) 42** ± 0·3	(14) 231** ± 20·4
			−3	(21) 40 ± 0·4	(21) 114 ± 10·2

Alternating R: 5 days +1° C. alternating with 5 days −0·6° C.
„ A: 7 days +1° C. „ „ 3 days −3° C.
„ B: 5 days +1° C. „ „ 5 days −3° C.
„ C: 3 days +1° C. „ „ 7 days −3° C.

Means marked with a single and double asterisk respectively are different from the mean immediately below at 5% and 1% level respectively.

may be postulated: (1) a slow decrease in the rate of vernalisation when the seeds are transferred from $+1^\circ$ C. to -3° C. but a rapid recovery to the former rate when brought back to $+1^\circ$ C.; (2) an increase of the process of vernalisation beyond the normal rate at $+1^\circ$ C. as a consequence of the transfer from -3° C. to $+1^\circ$ C. and a later return to the normal rate of $+1^\circ$ C.: (3) a combination of both effects.

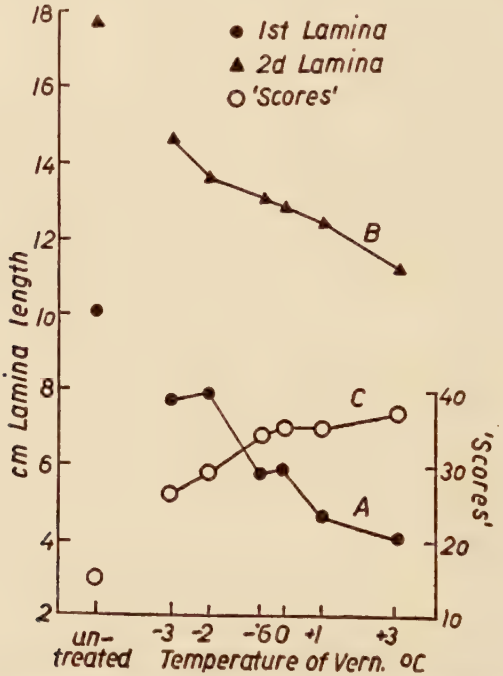

FIG. 2. The influence of different temperatures during vernalisation of grains of Petkus winter rye on subsequent progress to flowering ('scores', C) and the final length of the 1st (A) and 2nd (B) lamina when grown under 17 hrs. day-length. Duration of vernalisation 45 days. Data recorded after 49 days of growth.

THE LENGTH OF THE LAMINAE OF THE FIRST AND SECOND LEAF AFTER DIFFERENT TEMPERATURES AND TIMES OF COLD TREATMENT

1. *Winter rye*

In the first experiment it was found that the laminae of the first and second leaf both grew shorter after vernalisation by positive than by negative temperatures. Measurements showed (Table VII, Fig. 2) a reduction of the lamina length nearly proportional to the increase of the vernalising temperatures from -3° C. to $+3^\circ$ C. Vernalisation by -3° C. still caused a remarkable shortening of the leaf blades when compared with untreated seeds. The dependence of the length of the first and second lamina on the temperature during vernalisation was found to hold also for King II winter rye vernalised 104 days, a variety very similar to Petkus winter rye in its cold requirement. A prolongation of the cold treatment by the same temperature caused also

further shortening of the leaf blades in both varieties (Table VII). On the other hand, the decrease of vernalising temperatures from $+3°$ C. to $-3°$ C. was accompanied by a decrease of 'scores' (Table III, Fig. 2).

TABLE VII

Influence of Vernalising Temperatures from $+3°$ C. to $-3°$ C. on the Subsequent Length of the First and Second Lamina in Petkus and King II Winter Rye grown under a 17 hrs. Long Day (Length of Laminae in mm.)

Temperature of vernalisation °C.	Petkus winter rye*				King II winter rye*			
	Vernalised for 45 days		Vernalised for 76 days		Vernalised for 45 days		Vernalised for 104 days	
	1st lamina.	2nd lamina.	1st lamina.	2nd lamina.	1st lamina.	2nd lamina.	1st lamina.	2nd lamina.
+3	41	112	31	80	—	—	27	39
+1	47	124	—	—	41	102	29	64
0	59	128	—	—	—	—	—	—
−0·6	58	131	—	—	—	—	39	82
−2	79	136	—	—	—	—	—	—
−3	77	146	60	129	—	—	56	112
Untreated	101	177	105	181	49	161	91	159
m.s.d.								
$P = 0·05$	5·0	10·2	—	—	—	—	5·0	10·0
$P = 0·1$	6·5	13·0	—	—	—	—	6·5	14·0

* All measurements were done 49 days after planting. In the 45 days vernalised plants of Petkus Winter Rye laminae were also measured 28 days after planting. The mean difference between the corresponding means of the successive measurements was: $d = +1·64 \pm 0·77$ mm.

2. *Spring barley and winter barley*

When Kenia Spring Barley and Friedrichswerther Berg Winter Barley were vernalised by $-0·6°$ C. for 10, 20, 30, and 40 days, the two varieties responded differently to the cold treatment in 'scores' as well as in the length of the 1st and 2nd lamina (Table VIII and Fig. 3). In the spring variety there was a slight increase in 'scores' after 10 days of vernalisation only and a slight decrease in lamina length of the first leaf after 20 days' vernalisation only. In the winter variety, however, a clear progressive increase in 'scores' due to an extension of the cool treatment was accompanied by a clear gradual decrease of the length of the leaf blades. This experiment shows (1) that the reduction of the length of the first and second lamina is not necessarily a consequence of the extension of cold treatment as seen from the results with spring barley, and therefore (2) that a dependence of the lamina length on the degree of vernalisation in winter cereals is not impossible.

In winter rye (expt. I, Table I) the amount of growth during vernalisation was increasing with decreasing lamina length due to different temperatures. Therefore the shortening of lamina length by higher temperatures could be

assumed to be a direct response to the greater amount or rate of growth during vernalisation treatment. This relation, however, was not found in the barley experiments where the growth during the cold treatment was the same for the spring and winter variety, increasing from 1 mm. to 7 mm. from the shortest to the longest time of vernalisation.

TABLE VIII

The Influence of Different Duration of Cold Treatment on 'Scores' and the Length of the First and Second Lamina in Kenia Spring Barley and Friedrichswerther Berg Winter Barley grown under Natural Long Day. (Laminae in mm. measured 21 Days after Planting, Replicates per Treatment 36–39; 'Scores' estimated 66 Days after Planting, Replicates per Treatment 13–19)

Duration of vernalisation in days.	Kenia Spring Barley			Friedrichswerther Berg Barley.		
	'Scores.'	1st lamina.	2nd lamina.	'Scores.'	1st lamina.	2nd lamina.
0	52	104	194	15	140	216
10	56	103	197	18	112	189
20	55	94	192	31	103	181
30	56	93	201	40	95	175
40	56	94	199	46	85	168
m. s. d.						
$P = 0.05$	3·8	3·8	4·4	4·3	4·5	7·7
$P = 0.01$	5·2	5·0	5·9	6·2	6·1	10·3

These results suggest that this shortening of the first and second lamina is one of the features of the enhancement of the 'generative development' due to vernalisation in the same sense as it is claimed for the earlier completion of leaf growth (Denffer, 1939; Konovalov, 1944).

DISCUSSION

1. *The range of vernalising temperatures*

Results obtained by Purvis (1948) leave little doubt that vernalisation is equally effective for winter rye at all temperatures from +1° C. to +7° C., while at +10° C. the process of vernalisation is slower. Gassner (1918) showed that vernalising temperature of about +12° C. could still promote flowering. Temperatures fluctuating between +18° C. and 20° C. had no vernalising effect (Pojarkova, 1927), and as stated by Purvis (1948) +18° C. can already devernalise slightly. The upper limit of vernalising temperatures in winter rye is therefore somewhere between +12° C. and +18° C. The experiments described in this paper gave evidence that a rapid fall of the rate of vernalisation takes place between 0° C. and −4·5° C. and the lower limit of the vernalising process must be somewhere between −4·5° C. and −6° C. Even after a vernalisation of 94 days by −6° C. and also after a short-day treatment immediately after planting (16 days, 8-hour day-length) no hastening of spike differentiation could be induced as compared with corresponding control plants. Razumov, Feofanova, and Oleinkova (1948), however, claimed that

an extended vernalisation by −6° C. was effective in winter rye and more than in winter wheat. For Petkus winter rye the dependence of vernalisation on temperature may be represented by the curves shown in Fig. 4.

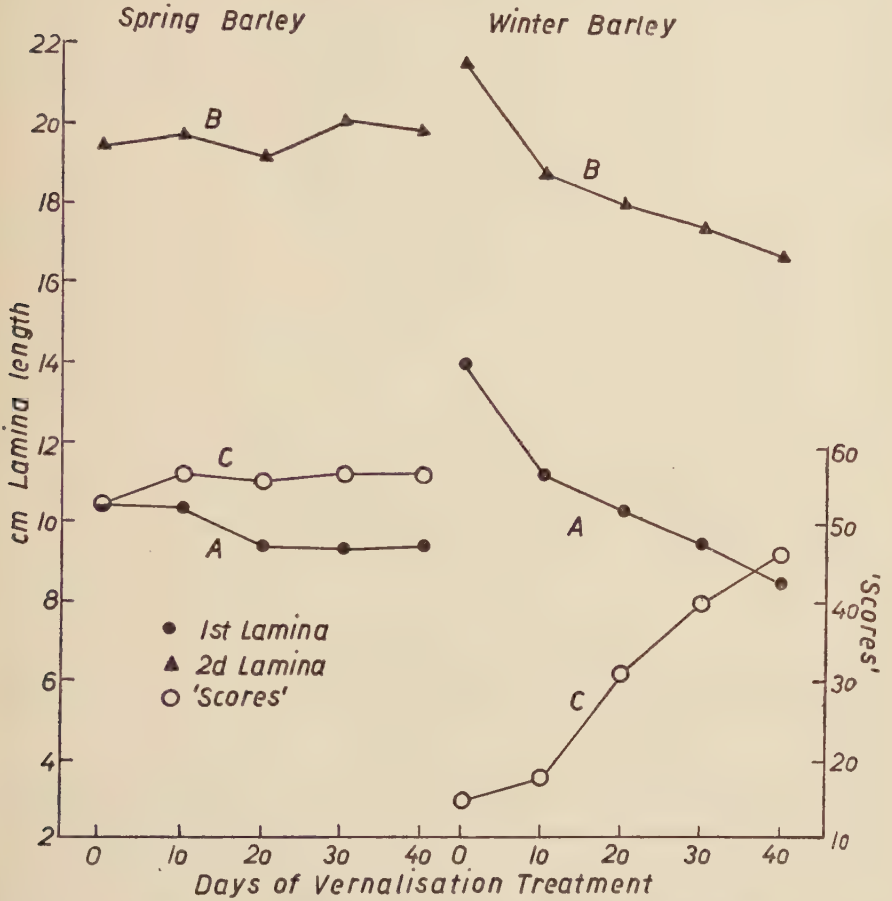

FIG. 3. The influence of different duration of cold treatment of germinated seed on 'scores' (C) and on the length of the first (A) and second (B) lamina in Kenia Spring Barley and Friedrichswerther Berg Winter Barley. Lamina measured 21 days, 'scores' estimated 67 days after planting. Date of sowing June 28, 1950.

The interpretation of this curve relating degree of vernalisation and temperature presents some difficulties. Purvis and Gregory (1952) have put forward evidence to show that the original scheme of the reactions concerned in vernalisation (Purvis and Gregory, 1937; Gregory, 1948) requires modification.

It was originally suggested that a precursor *A* is converted into an intermediate substance *B* under the influence of low temperature in the case of winter rye, and by a genetical mechanism independent of temperature in the

case of spring rye. The intermediate B is the substrate for the further reactions controlled by length of day leading to flower initiation. It now appears (Purvis and Gregory, 1952) from experiments on devernalisation by high temperature that in winter rye there is a further step $A \rightleftarrows A' \rightarrow B$, the step $A \rightleftarrows A'$ being reversible. The reaction $A' \rightarrow B$ can proceed both at low and high temperature, the Q_{10} of the reaction being greater than 2. The intermediate substance B' is thermostable, so that as vernalisation proceeds and B' accumulates the reversal of vernalisation by high temperature fails.

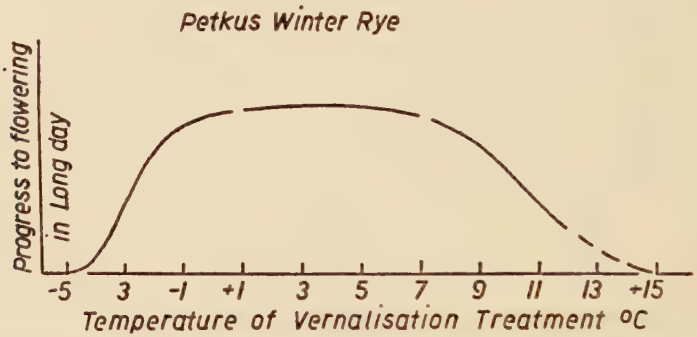

FIG. 4. The dependence of the progress to flowering under long days on the temperature of the preceding vernalisation treatment in Petkus winter rye.

In the experiments reported in this paper it is shown that vernalisation of winter rye fails if the temperature is reduced below $-4°$ C., and is progressively reduced as the temperature of vernalisation is reduced below 1° C. This might be due either to the progressive reduction of stage $A \rightarrow A'$ or stage $A' \rightarrow B$.

The transfer experiments (Table VI), however, show that interpolated periods of 3 days at $-3°$ C. following periods of 7 days at 1° C. has little effect on progress to flowering, whereas longer interpolated periods at $-3°$ C. alternating with shorter periods at 1° C. lead to progressive delay in flower initiation. These results might be interpreted as evidence that the reaction $A' \rightarrow B$ is slowed down at $-3°$ C. and ceases at $-6°$ C. altogether. Alternatively, of course, the primary reaction $A \rightleftarrows A'$ may itself be checked by the lower temperature. It is suggested (p. 426) that in consequence of transfer from $-3°$ C. to 1° C. the rate of vernalisation is increased during the period at 1° C. to a rate exceeding that 'normal' for this temperature. If the effect of $-3°$ C. is to reduce rate to the progress from $A' \rightarrow B$, then A' will accumulate and after transfer to 1° C. this reaction will temporarily increase in rate. Without further experiment it is not possible to decide this matter.

2. *Leaf growth as affected by cold treatment of germinated grains*

Thimann and Lane (1938) found a shortening of the first leaf by vernalisation of winter wheat. Denffer (1939) obtained the same effect in winter barley

and showed that the rate of growth of leaves was increased by vernalisation. In both experiments no spring varieties were included. Purvis (1934) found that the rate of leaf production up to flower initiation was the same in vernalised and unvernalised winter rye. Konovalov (1944) claimed that the successive emergence of leaves was more rapid with than without vernalisation and each leaf reached its maximum size sooner after vernalisation.

In the experiments described in this paper a marked increase of the length of first and second laminae resulted on lowering the vernalising temperatures from $+3°$ C. to $-3°$ C. in Petkus and in King II winter rye. A prolongation of vernalisation at the same temperature also induced further shortening of lamina length. All these effects might be interpreted as simple temperature effects on the embryo and its different amount of growth during the cold treatment without relation to the process of true vernalisation which occurred simultaneously in the winter varieties. Kenia Spring Barley treated like Friedrichswerther Berg Winter Barley showed as a whole neither a progressive reduction of the first and second lamina nor a hastening to flowering when compared with winter barley as seen in Fig. 3. This shows that the shortening of the first and second lamina is not a necessary consequence of the cold treatment of the embryo. Nor is it due to the amount or rate of leaf growth during vernalisation which was the same for the spring and winter variety. The experiments with the winter varieties suggest therefore that the internal change of the embryo produced by vernalisation treatment expresses itself in subsequent shortening of the first and second lamina.

Acknowledgements

The experiments described were carried out at the Plant Breeding Institute, School of Agriculture, Cambridge, and the vernalisation treatments at the Low Temperature Station, Cambridge. The author wishes to express his gratitude to Dr. D. H. Bell for helpful consultation and for facilitating this work, and to thank Professor F. G. Gregory, F.R.S., for many valuable discussions.

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Dimorphism and Monomorphism in the Plumbaginaceae

II. Pollen and Stigmata in the Genus *Limonium*

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With eight Figures in the Text

Abstract

A survey of pollen- and stigma-dimorphism and monomorphism in the genus *Limonium* (as understood at present) reveals considerable heterogeneity which is useful for the solution of taxonomic, phylogenetic, and phytogeographic problems.

Two main pollen types (A and B) and two main stigma types (*cob* and *papillate*) occur in the genus. Capitate stigmata also occur.

'Type A' pollen has frequently been found in conjunction with 'papillate' stigmata as a secondarily monomorphic (self-compatible) combination and this must have arisen at least four times in the genus as well as in *Armeria*. 'Type B' pollen and 'cob' stigmata are recorded together for the first time (also as a secondarily monomorphic, self-compatible combination) in *L. echioides*. Apomixis, already discovered in subsections Densiflorae and Dissitiflorae, has been found in *L. cosyrense* of the subsection Steirocladae.

The taxonomic and phylogenetic significance of the facts in discussed.

Introduction

A SURVEY of the distribution of pollen- and stigma-dimorphism among the genera of the Plumbaginaceae has been published in the first paper of this series (Baker, 1948) and the relationship between such dimorphism and the well-known phenomenon of heterostylism has been demonstrated. In the paper referred to it has been shown that pollen-dimorphism is a feature of the tribe Staticeae, only the genus *Aegialitis* failing to show it. Stigma-dimorphism is also to be seen in the genera *Limonium*, *Limoniastrum*, and *Armeria*. *Goniolimon* and *Acantholimon* possess capitate stigmata which are monomorphic, and there are species at present retained within the genus *Limonium* which also possess capitate stigmata.

It is the purpose of the present paper to present a survey of dimorphism and monomorphism within the genus *Limonium* Mill. as it is at present understood. From this survey preliminary conclusions will be drawn regarding the value of these pollen and stigma characters in taxonomy. In a subsequent paper correlations between reproductive method and geographical distribution will be sought. In these connexions it is noteworthy that there is no satisfactory taxonomic treatment of the genus in existence. The most recent attempt at a

[Annals of Botany, N.S. Vol. XVII, No. 67, July, 1953.]

comprehensive treatment is already over 100 years old and is that made by Boissier (1848) for De Candolle's 'Prodromus'. Since then Bentham and Hooker (1873–6) have divided the genus into a smaller number of sections, while Pax (1897) has created a trio of subgenera, although his general arrangement of the sections is very similar to that of Boissier (see Table I). Several subsequent authors (e.g. Wangerin, 1911; Blake, 1916, 1923) have been concerned with certain subsections of the genus or with geographically limited fragments of these, but no further general work has appeared.

None of these classificatory schemes approaches perfection and there will need to be fairly considerable revision in the future. Boissier's is the most detailed scheme and the only one in which a published attempt has been made to place all the known species of the time (for many of which he was the discoverer). Since that great taxonomist drew up his classification, however, species which must be referred to very distinct additional sections (*Pterolimon* Hook. and *Arthrolimon* F.v.M.) have become known. The section Schizopetalum was created by Boissier himself at a later date (Boissier, 1854–9) to accommodate another group of species.

TABLE I

Tabulation of Classifications of the Genus Limonium (*then known as Statice*)

Boissier (1848).	Bentham and Hooker (1873–6).	Pax (1897).
Genus *Statice*	Genus *Statice*	Genus *Statice*
Sect. *Pteroclados*	Sect. *Schizopetalum*	Subgen. *Limonium*
Subsect. Odontolepidae		Sect. *Pteroclados*
Subsect. Nobiles		Sect. *Ctenostachys*
Sect. *Ctenostachys*	Sect. *Goniolimon*	Sect. *Plathymenium*
Sect. *Plathymenium*	(incl. *Circinaria*)	
Subsect. Rhodantheae		Sect. *Eu-limonium*
Subsect. Chrysantheae		
Sect. *Limonium*		Sect. *Sphaerostachys*
Subsect. Genuinae	Sect. *Limonium*	Sect. *Jovibarba*
Subsect. Densiflorae		
Subsect. Dissitiflorae		Sect. *Circinaria*
Subsect. Steirocladae		
Subsect. Hyalolepidae	Sect. *Siphonantha*	Subgen. *Siphonantha*
Subsect. Sarcophyllae	(incl. *Polyarthrion*,	
Sect. *Sphaerostachys*	*Myriolepis*,	Sect. *Eusiphonantha*
Sect. *Jovibarba*	*Eurychiton*)	(incl. *Eurychiton*)
Sect. *Schizhymenium*		Sect. *Psylliostachys*
Sect. *Circinaria*		
Sect. *Polyarthrion*	Sect. *Psylliostachys*	
Sect. *Myriolepis*		Sect. *Pterolimon*
Sect. *Siphonantha*		
Sect. *Psylliostachys*	Sect. *Pterolimon*	Subgen. *Schizopetalum*
Genus *Goniolimon*		Genus *Goniolimon*

It is notable that even Boissier, when grouping the appropriate species of *Limonium* in his 'Flora Orientalis' (Boissier, 1879), makes no reference to his subsections of the 'section Limonium' and there is good evidence that they are

sometimes circumscribed unnaturally or unnecessarily. They are likely to have polyphyletic origins, for hybridization in the genus seems often to be possible between species which are not very close morphologically.

However, Boissier's original scheme serves best as the framework for the presentation of the results of this survey. The absence of a thoroughly satisfactory classification is a disadvantage as far as concise presentation is concerned, but there is, on the other hand, an advantage in that the subsequent treatment of the genus by a taxonomist (which it is hoped will not be long delayed) will be materially assisted by the demonstrations of relations and reproductive methods given here.

The situation in the subsections Densiflorae and Dissitiflorae which contain numerous apomictic species is discussed elsewhere (Baker, 1950).

Justification may be needed for the use of the generic name *Limonium* in preference to the equally well-known *Statice* (which has been applied variously to the sea-lavenders and to the thrifts). This matter has been dealt with in detail by Lawrence (1940, 1947), who has shown that *Statice* must be rejected as a *nomen ambiguum*. This decision was made by the International Committee on Nomenclature which has conserved Miller's name *Limonium* for the sea-lavenders and Willdenow's name *Armeria* for the thrifts. As yet, not all specific names have been transferred legitimately.

Materials and Methods

In this survey use has been made of fresh material collected in Europe and North America as well as of preserved material contained in the principal herbaria of Great Britain and the U.S.A. A list of the herbaria consulted is given in Table II, and it is through the generous co-operation of the keepers of these institutions that pollen- and stigma-analyses have been made on individual flowers removed from appropriate specimens. In all, some 4,000 determinations have been made by the methods already described (Baker, 1948).

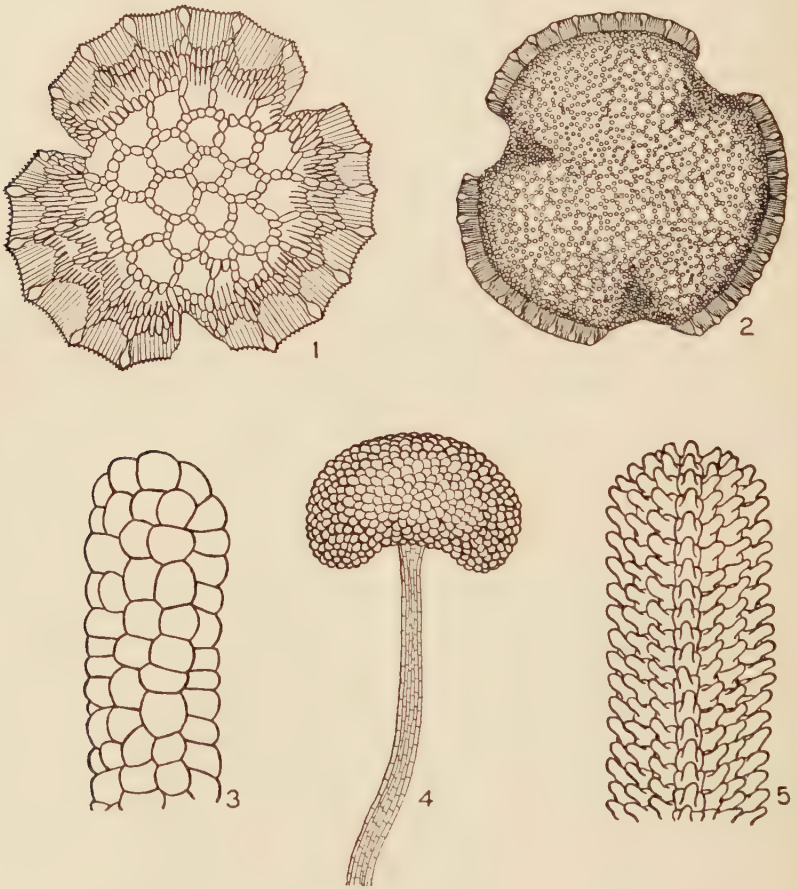
Table II

Herbaria consulted

Great Britain.	U.S.A.
Royal Botanic Gardens, Kew	National Herbarium, Washington, D.C.
British Museum (Natural History)	Gray Herbarium, Harvard University
Royal Botanic Garden, Edinburgh	Bailey Hortorium, Cornell University
National Museum of Wales, Cardiff	Dudley Herbarium, Stanford University
Yorkshire Museum	University of California, Berkeley
Manchester Museum	California Academy of Sciences, San Francisco
Druce Herbarium, Oxford University	Washington State College, Pullman, Wash.
Cambridge University	
Leeds University	

In a number of cases, where it has been possible to cultivate material at the Experimental Garden of the University of Leeds, it has been possible to

arrive directly at conclusions concerning the self-compatibility or self-incompatibility of the various combinations (and, in some cases, to prove the occurrence of apomixis). In these cases, too, it has been possible to make chromosome-counts which have helped in the assessments of relationships.



FIGS. 1–5.

FIG. 1. Type A pollen. (×800.) FIG. 2. Type B pollen. (×800.) FIG. 3. Portion of 'cob' stigma. (×300.) All from *Limonium vulgare*, Patrington Haven, E. Yorks. FIG. 4. Portion of style and capitate stigma from *L. griffithii*, Khyber Pass (K 1335). (×80.) FIG. 5. Portion of 'papillate' stigma from *L. vulgare*, Patrington Haven, E. Yorks. (×300.)

RESULTS

The two types of pollen found in dimorphic taxa are illustrated in Figs. 1 and 2. These types ('A', Fig. 1; 'B', Fig. 2) have already been described in detail (Baker, 1948). They are found almost throughout the genus with only slight deviations from the typical appearance. A number of dimorphic species possess capitate stigmata (cf. Fig. 4) that are indistinguishable whichever of

the two pollen types they accompany. The remaining majority of the dimorphic species show correlated stigma-dimorphism, 'cob' stigmata (Fig. 3) being found in flowers producing Type A pollen and 'papillate' stigmata (Fig. 5) occurring in flowers producing Type B pollen. Monomorphic species show a variety of combinations of pollen and stigmata. The available evidence is unanimous that those secondarily monomorphic species which are not apomictic are self-compatible and all but one of these combine Type A pollen with 'papillate' stigmata. The combination of Type B pollen with 'cob' stigmata has been encountered only in *Limonium echioides* (p. 443).

For convenience, the detailed results have been grouped together to form Table III.

TABLE III

Pollen and Stigma Types in Species of Limonium

Section.	Subsection.	Species examined.	Result { P. = pollen S. = stigmata.	Distribution.
Pteroclados	Odontolepidae	*L. sinuatum*	Dimorphic P. & S.	Mediterranean
		L. bonduellii	,,	Algeria
		L. thouinii	,,	Middle East to Canaries
	Nobiles	*L. arborescens*	Dimorphic P. & S.	Canary Isles
		L. macrophyllum	,,	,,
		L. brassicifolium	,,	,,
		L. puberulum	,,	,,
		L. bourgaeii	,,	,,
		L. macropterum	,,	,,
		L. imbricatum	,,	,,
		L. preauxii	,,	,,
		L. perezii	,,	,,
Ctenostachys	—	*L. mucronatum*	Dimorphic P. & S.	Morocco
		L. pectinatum	,,	Canary Isles
		L. papillatum	,,	,,
		L. humboldtii	,,	,,
		L. corculum	,,	,,
		L. brunneri	,,	Cap Verde Isles
		L. braunii	,,	,,
Plathymenium	Rhodantheae	*L. flexuosum*	Dimorphic P. & S.	Baikal region
		L. congestum	?Monomorphic, Type B pollen	Altai region
		L. tenellum	Dimorphic P. & S.	Mongolia–Gobi
		L. nudum	,,	Middle East
	Chrysantheae	*L. aureum*	Dimorphic P. & S.	Dahuria–Mongolia
		L. chrysocomum	,,	Songoria
		L. schrenkianum	,,	,,
		L. bicolor	,,	N. China
		L. xantholeucum	,,	Outer Mongolia
		L. wrightii	,,	Formosa–Riu Kiu Isles
		L. sinense	,,	China, Formosa
		L. japonicum	Monomorphic, A/papillate	Korea, Japan
		L. australe	,,	Queensland–Tasmania
Limonium	Genuinae	*L. vulgare* (incl. var. *macrocladon*)	Dimorphic P. & S. (and male sterile)	Europe, N. Africa, Azores, Asia Minor
		L. gmelinii (incl. vars.)	,,	Caucasus–Siberia
		L. meyeri	,,	Tauria, Trans- and Cis-Caucasia, Attica
		L. tomentellum	,,	Siberia

TABLE III—*continued*

Section.	Subsection.	Species examined.	Result {P. = pollen S. = stigmata.}	Distribution.
Limonium	Genuinae	*L. effusum*	Dimorphic P. & S. (and male sterile)	Lydia
		L. brasiliense	,,	Brazil–Patagonia
		L. uruguayense	,,	Uruguay
		L. patagonicum	,,	Patagonia
		L. guaicuru	,,	Chile
		L. mexicanum	Monomorphic, A/papillate	Baja, California
		L. californicum	,,	California and Nevada
		L. limbatum	,,	New Mexico and Texas
		L. endlichianum	,,	NE. Mexico
		L. angustatum	,,	Texas–Florida
		L. obtusilobum	,,	Florida
		L. carolinianum	,,	NE. Mexico–N.Carolina
		L. nashii	,,	NE. Mexico–S. Carolina
		L. trichogonum	,,	New Jersey–S. Labrador
		L. lefroyi	,,	Bermuda
		L. humile	,, (and male sterile)	N. Atlantic Europe
	Densiflorae	Sexual species (Dimorphic P. & S.) and apomictic species		
	Dissitiflorae	Sexual species (Dimorphic P. & S.) and apomictic species		
	Steirocladae	*L. virgatum**	Dimorphic P. & S.	Mediterranean–Asia Minor
		L. dictolcladum	,,	Mediterranean
		L. dichotomum	,,	Sicily and Corsica
		L. articulatum	,,	Corsica
		L. minutum (and var. *microphyllum*)	,,	NW. Mediterranean
		L. cosyrense	Apomictic, A/cob	Pantellaria and Sicily
		L. cordatum	Dimorphic P. & S.	Mediterranean
		L. cumanum	,,	W. Italy
		L. linifolium	,,	Cape of Good Hope
		L. kraussianum	,,	,,
		L. equisetinum	,,	,,
		L. dregeanum	,,	,,
		L. scabrum	,,	,,
	Hyalolepidae	*L. latifolium*	Dimorphic P. & S.	S. Russia–Bulgaria
		L. bellidifolium	,,	Europe–Songoria
		L. decipiens	,,	Altai–Songoria
		L. dubyi	,,	S. France and Spain
		L. pruinosum	,,	Egypt, Arabia, &c.
		L. perfoliatum	,,	Persia–Afghanistan
		L. asparagoides	,,	Algeria, Morocco
		L. tuberculatum	,,	W. Tropical Africa
		L. mauritanicum	,,	,,
		L. bahamense	,,	Bahamas
		L. haitiense	Monomorphic, A/papillate	Haiti
	Sarcophyllae	*L. suffruticosum*	Dimorphic P. & S.	S. Russia–Songoria
		L. axillare	,,	Red Sea countries–Egypt
		L. stocksii	,,	Baluchistan and Sind–Egypt
		L. arabicum	,,	E. Arabia
		L. cylindrifolium	,,	Arabia
		L. somalorum	,,	Somaliland
Sphaerostachys	—	*L. globuliferum*	Dimorphic P. & S.	Asia Minor
Jovibarba	—	*L. jovibarbum*	Dimorphic P. & S.	Cap Verde Isles

* Some 'varieties' of this species may not be dimorphic.

TABLE III—*continued*

Section.	Subsection.	Species examined.	Result {P. = pollen S. = stigmata.}	Distribution.
Schizhymenium	—	*L. echioides*	Monomorphic, male sterile and B/cob	Asia Minor–Iberian peninsula
		L. cabulicum	Dimorphic pollen/ capitate stigmata	Punjab and Khyber
		L. owerinii	,,	Armenia
Circinaria	—	*L. purpuratum*	Monomorphic, B/ capitate stigmata	Cape of Good Hope
		L. roseum	,,	,,
		L. amoenum	,,	,,
		L. capense	,,	,,
Polyarthrion	—	*L. caesium*	Dimorphic P. & S.	E. Spain
		L. ornatum	,,	Morocco
Myriolepis	—	*L. ferulaceum*	Dimorphic P. & S.	Mediterranean
		L. diffusum	,,	,,
Siphonantha	—	*L. tubiflorum*	Dimorphic P. & S.	Alexandria
Psylliostachys	—	*L. spicatum*	Dimorphic P. & S.	Caspian–Altai
		L. suworowii	,,	NE. Persia–Turkestan
		L. alberti	,,	Turkestan
		L. leptostachyum	,,	S. Persia
Schizopetalum	—	*L. macrorhabdon*	Dimorphic pollen/ capitate stigmata	Tibet, Kashmir, Baltistan
		L. gilesii	,,	Gilgit
		L. griffithii	,,	Afghanistan
		L. xipholepis	Dimorphic P. & S. (see text)	Somaliland
Pterolimon	—	*L. plumosum*	Monomorphic. 'Close-meshed Type A'/capitate stigmata	Chile
		L. peruvianum	,,	Peru
Arthrolimon	—	*L. salicornaceum*	Monomorphic. 'Close-meshed Type A'/ capitate stigmata	Western Australia
Goniolimon	—	*G. tataricum*	Dimorphic pollen/ capitate stigmata	SE. Europe–Siberia
		G. callicomum	,,	Songoria
		G. speciosum	,,	S. Russia–Mongolia
		G. eximium	,,	Chinese Songoria
		G. kaufmannianum	,,	Turkestan
		'G. incanum'	,,	
		'G. dumosum'	,,	

DISCUSSION

1. *Species with capitate stigmata*

The species with capitate stigmata have been placed in six sections of the old genus *Statice* (although *Goniolimon* was given separate generic status by Boissier) and, indeed, they are a most heterogeneous collection.

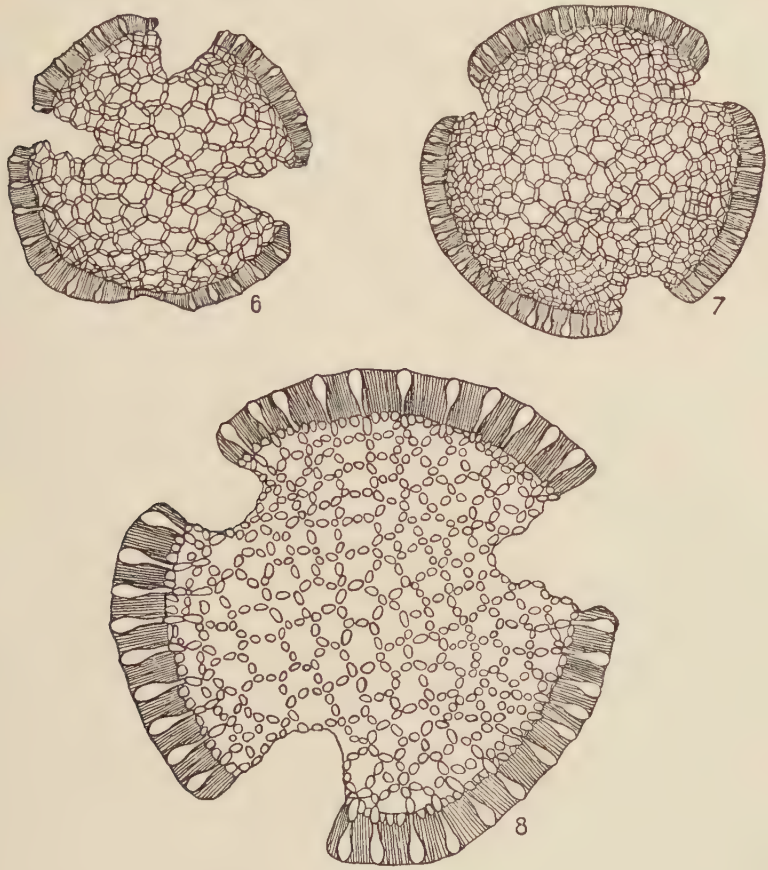
Section Pterolimon. This section contains an extremely odd pair of closely related species, *Limonium plumosum* (Phil.) Kuntze and *L. peruvianum* Kuntze. They are shrubs with narrow, scale-like leaves and spicate inflorescences. The

calyx, in each case, consists of a short tube, the five nerves of which end in feathery awns, the like of which are not to be seen elsewhere in the genus. They are desert plants, *L. plumosum* being found in the provinces of Antofagasta and Atacama in northern Chile, while *L. peruvianum* occurs in similar habitats in Peru. Their sub-tropical distribution, their shrubby habit, and their very distinct floral morphology indicate that they are not closely related to any other group (certainly not to the section *Limonium* which, alone of the others, is to be found in South America). This is strikingly confirmed by the pollen picture. The kind of pollen found is not met with in any of the groups known to Boissier, being matched only by Baron von Mueller's equally odd section *Arthrolimon* from Western Australia. The ornamentation of the tricolpate grains (Fig. 6) consists of ridges made up of partially fused rods, as in Type A of the dimorphic species. The mesh formed by the reticulate arrangement of ridges, however, is very much closer than in the dimorphic species and produces a distinctive appearance. The grains are quite uniform in size and some have been seen adhering to the capitate stigmata.

The extraordinarily disjunct occurrence of this pair of species, far from the present-day centre of variability of *Limonium* (the Mediterranean) or even the Old World as a whole (where the majority of the species is to be found), may indicate that they are the last remnants of a line of specialization indulged in by the ancestors of *Limonium* which is now extinct except for themselves. The union of this section with *Eusiphonantha* and *Psylliostachys* into a single subgenus, as suggested by Pax (1897), is quite untenable and was based simply on the greater than usual union of the petals.

Section Arthrolimon. Very little is known about these Western Australian species, although it is to be hoped that the publication of this survey will stimulate interest in the collection of material for growing these plants. *L. salicornaceum* (F. v. M.) Kuntze appears to lack leaves and the stems have the articulate appearance of a *Salicornia*. It grows at Shark's Bay on the extreme west coast of the continent and in the Abrolhos Islands. '*Statice Macphersoni*' (from saline soil near Coolgardie, over 300 miles inland from the west coast) has the unusual distinction of having been described first in the 'Chemist and Druggist of Australasia', and it has not been possible to see herbarium material of this species. Even Baron F. von Mueller, who described it (von Mueller, 1895), saw no more than a leafless flowering-shoot which, however, was sufficient to indicate its relationship with his other species, *L. salicornaceum*. The calyx of the latter species is remarkable in its riblessness and the corolla forms a long tube, while in the former the lobes of the corolla are longer than the tube. *L. papillatum* (Webb. et Berth.) Kuntze (section *Ctenostachys*) which is found in the Canary Islands and a few of the species of the sections *Plathymenium*, *Myriolepis*, and *Polyarthrion* and the subsection Steirocladae have habits superficially resembling that of *L. salicornaceum*. All of these, however, have pollen and stigmata of the normal types, quite unlike those found in *Arthrolimon*, and the superficial resemblances can be attributed to convergent evolution.

As pointed out above, there is a striking similarity between the pollen of *L. salicornaceum* (Fig. 7) and that of the two species of the section *Pterolimon*, although the macroscopic morphologies of the species are quite distinct. Both sections share an articulate habit and a tendency to leaflessness, however, and



FIGS. 6–8.

FIG. 6. Pollen grain of *Limonium plumosum*, Atacama, Chile (N 60). (×800.) FIG. 7. Pollen grain of *L. salicornaceum*, Shark's Bay, W. Australia (K 644). (×800.) FIG. 8. Pollen grain of *L. purpuratum*, between Brandt Vlei and Rooihoogte, Cape Province, S. Africa (K 331). (×800.)

there are some similarities in their corollas. It is possible that these sections are divergent representatives of the same very primitive stock in the Staticeae. von Mueller himself (1878) placed his new section alongside what he referred to as the section 'Petrolimon'. The stigmata of the West Australian species are not so obviously capitate as those of *L. plumosum* or *L. peruvianum*, but this may only emphasize the divergence which has been produced after isolation.

Section Circinaria. The monomorphism shown by this strictly South African section (of four species) is not exactly matched elsewhere in the genus even when it is considered in its widest sense. The very large flowers produce exceptionally large grains and these have a very regular polygonal pattern of rather coarse spines (Fig. 8). The general appearance of the grains is intermediate between that of the last two sections and that of 'Type B' grains of a dimorphic species. The stigmata are consistently capitate and the upper parts of the styles are coiled. The monomorphism cannot be definitely classified as primary or secondary, for we know nothing of any reasonably close relatives of this morphologically rather isolated group. Because of their capitate stigmata, Bentham and Hooker (1873–6) included these species in the genus *Goniolimon*, but this settlement is too obviously artificial to be satisfactory, for there is no close general morphological resemblance. Pax (1897) included *Circinaria* (as a section) within the subgenus *Limonium*, but there is little relationship between these semi-shrubby species and the other South African representatives of the genus (which are dimorphic herbs of Boissier's subsection Steirocladae). Geographical restriction and isolation again appear to be correlated with morphological divergence and we may, once more, be justified in regarding the species of this section as the contemporary representatives of a line which diverged from the common stock of the tribe Staticeae a long time ago. For this reason the resemblance to the sections *Pterolimon* and *Arthrolimon* in pollen and stigma morphology may not be in any way coincidental.

Sections Schizopetalum and Schizhymenium. The section *Schizopetalum* was created by Boissier (1854–9) to accommodate the species which is now called *Limonium macrorhabdon* (Boiss.) Kuntze and whose distribution is from Tibet to Baluchistan. Particularly important among the characters which he used to distinguish the section was the bifid nature of the limbs of the petals. Other species have been discovered since then which have shown this petal character to have no sectional significance. Thus there can be little argument about placing the morphologically very similar *L. gilesii* (Hemsl.) H. G. Baker (inedit.) (from Gilgit) in the same section in addition to *L. griffithii* (A. & H.) Kuntze which was described from plants collected in Afghanistan (Aitchison, 1882). *L. cabulicum* (Boiss.) Kuntze, which Boissier (1848, 1879) referred to his section *Schizhymenium* after examining very scanty material, is extremely close to *L. griffithii* in habit (see also Aitchison, 1882). The rosettes and inflorescences of this Afghanistani species are also very like those of *L. macrorhabdon*. This and the general agreement in pollen- and stigma-characters of all these species suggests that the two sections, *Schizopetalum* and *Schizhymenium*, might well be united. Indeed, in the 'Flora Orientalis' Boissier (1879) placed them alongside.

Of the two remaining species of the old section *Schizhymenium*, the abundant *L. echioides* (L.) H. G. Baker (inedit.) stands apart and merits separate consideration (see p. 443). *L. owerinii* (Boiss.) Kuntze, on the other hand, is poorly represented in herbaria. Boissier (1848, 1879) seems to have had access only

to a single specimen of this probable annual and, because it possessed a facies similar to that of *L. echioides*, he placed it alongside that species. The single Armenian representative in the herbarium at Kew seems utterly unlike *echioides* but, unfortunately, this specimen lacks leaves. It may be significant, however, that a specimen of *L. cabulicum*, collected by Griffith (No. 1590), attested by Boissier, and now lodged in the same herbarium, has a scape with much the same appearance as that of *L. owerinii*. It appears to be etiolated. The 'hammer-head' stigmata of *L. owerinii* are quite comparable with those illustrated in Aitchison's (1882) account of *L. griffithii* (see also Fig. 4) and supports the other claims for the union of the two sections.

A drawing of the styles of *L. cabulicum* on a herbarium-sheet (Griffith, from near Karabagh, Afghanistan) at Kew gives the impression of fat but linear stigmata. This is misleading because the capitate stigmata, easily seen when dried flowers are carefully dissected after boiling in water, protrude from the corolla in young flowers and most have been lost from the material figured on the herbarium-sheet.

Limonium xipholepis (J. G. Baker) Hutch. & Bruce was described by J. G. Baker (1895) from material collected in Somaliland by Miss E. Cole. After his description he placed the species 'Near *S. macrorhabdos* Boiss. and *S. Griffithii* Aitch. et Hemsl.' This would indicate membership of the section *Schizopetalum*. The type specimen and other plants preserved at Kew have been examined; they have dimorphic linear stigmata and dimorphic pollen. Consequently this species cannot be placed along with the two species just mentioned. Morphologically there is considerable resemblance between *L. xipholepis* and *L. carinense* (Chiov.) H. G. Baker (inedit.) which is another Somali species with linear stigmata.

In his description of *L. macrorhabdon* (as *Statice macrorhabdos*) Bossier (1854–9, p. 68) uses the following words: 'Species curiosissima foliis *Limonium* et inflorescentia *Acantholimonis* donata . . .' and later, 'Inflorescentia *Acantholimonis* sect. *Glumariae*'. It may be that in a form resembling the species of this section we may see a common ancestor of the genera *Limonium* and *Acantholimon* (the latter genus also showing dimorphic pollen and capitate stigmata). Compared with *Limonium* (sensu lato) *Acantholimon* has a relatively restricted distribution which includes the geographical range of *Schizopetalum* and *Schizhymenium* together. This matter will be discussed more fully when chromosome-counts are available.

In the above treatment mention of *L. echioides* has been deferred. This is by far the commonest species among those placed by Boissier in his section *Schizhymenium* (where he took no account of stigma shape). *L. echioides* is out of agreement with the previous species in many points of morphology and disagrees strikingly in its stigmata, which are linear. All plants examined, from the entire range of the species, have either Type B pollen or are male-sterile and all possess 'cob' stigmata. Such a combination of pollen and stigmata should produce self-compatibility and this will be tested experimentally. It has not been encountered elsewhere. This species will have to be

transferred from association with *L. cabulicum* and *L. owerinii* to some other section.

Genus Goniolimon. This almost exclusively Asiatic genus was created by Boissier (1848) and separated from '*Statice*' by its capitate stigmata. From his descriptions of the genera it seems that it was only in *Goniolimon* that Boissier realized the presence of non-linear stigmata. It is likely that, had he known of the capitate condition of the stigmata in other groups, he would have separated them too. *Goniolimon* was re-submerged in '*Statice*' by Bentham and Hooker (1873–6) but was reconstituted by Pax (1897).

Maury (1886) presented evidence of close anatomical similarity between *Goniolimon* and *Limonium* and claimed that this argued for their union. The capitate stigmata speak against this, but fairly close relationship is not impossible if it is assumed that *Goniolimon* stands near to the point of departure of groups with linear stigma from the capitate-stigma stock. All species of *Goniolimon* appear to possess dimorphic pollen.

2. *Species with linear stigmata*

The sections containing species with linear stigmata show very little variation in details of pollen- and stigma-morphology. This, together with the reasonable uniformity of other floral morphology, suggests that they represent a fairly homogeneous group: a conclusion which is in no way invalidated by the great range of vegetative diversity. Secondary monomorphism appears to have arisen at least four times (sect. *Plathymenium*, subsect. Chrysantheae; sect. *Limonium*, subsects. Genuinae and Hyalolepidae; *L. echioides*) and seems to reflect the usual self-compatibility in each case.

Limonium cosyrense (Guss.) Kuntze (sect. *Limonium*, subsect. Steirocladae) has shown only Type A pollen and cob stigmata, a typically self-incompatible combination. The pollen is irregular in size. Such a pollen- and stigma-picture has been shown to characterize apomictic species (Baker, 1950), and this produces a strong suspicion that *L. cosyrense* is also apomictic. Confirmation is provided by the triploid chromosome count ($2n$–27) given by the seed-fertile material of this species obtained from the Royal Horticultural Society's garden at Wisley. This is the first demonstration of apomixis outside the subsections Densiflorae and Dissitiflorae (cf. Baker, 1950) and suggests that the taxonomic position of *L. cosyrense* relative to these subsections needs reconsideration.

Conclusion

Correlated pollen- and stigma-dimorphism (together with the associated incompatibility system) results from the actions of a mutually adapted group of genes which are inherited together. Such a complex system is unlikely to arise repeatedly in nature and, therefore, probably provides a worthwhile guide to phylogeny. We may assume, with some confidence, that the species with linear, dimorphic stigmata (including those of the genera *Armenia* and *Limoniastrum* as well as most of *Limonium*) can be traced back to a common

ancestor which was *not* ancestral to the species with capitate stigmata. Similarly those species with capitate stigmata and dimorphic pollen probably have a remote common ancestor which is probably not shared by the species with capitate stigmata but distinctive kinds of monomorphic pollen.

Consequently it is unreasonable to ask a single genus *Limonium* to contain species with capitate stigmata and various kinds of pollen (representing one broad evolutionary line in the Staticeae, including *Aegialitis* and *Acantholimon*) and at the same time the species with linear stigmata (agreeing with *Armeria* and *Limoniastrum* in another line). A satisfactory taxonomic treatment will involve the creation of additional genera.

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Experimental and Analytical Studies of Pteridophytes

XXI. Investigations on *Marsilea*

3. The Effect of Various Sugars on Development and Morphology

BY

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With Plates XXV and XXVI and six Figures in the Text

Abstract

An account is given of the effects of various concentrations of glucose, sucrose, and fructose on the development and morphology of aseptic cultures of *Marsilea* sporelings. The response of the sporelings to a range of different sugars and to growth in liquid or on solid media is also described.

The results are discussed with special reference to the relationship between nutrition and heteroblastic leaf development. Other aspects of the influence of various sugars on the growth of plants in aseptic culture are considered in relation to the existing literature.

Introduction

PREVIOUS investigations on the growth of *Marsilea* in aseptic culture (Allsopp, 1952, 1952*a*) have provided support for the view of Goebel (1898, 1928) that the heteroblastic development of many plants is to be referred to the increasing nutritional capacity of the enlarging germling. The results already published show that in *Marsilea* reversion to juvenile stages may be induced by restriction of the organic or mineral nutrients of the culture medium; also by various surgical treatments designed to limit the supply of nutrients to the growing-apex. The present paper deals with the effect on heteroblastic development and general morphology of changes in the nature and concentration of the sugar supply.

Materials and Methods

Unless otherwise indicated, the experimental work was carried out on *Marsilea Drummondii* A. Br. *M. vestita* Hook. and Grev. was occasionally used.

Sterile cultures were obtained by the technique described in the first paper of the series (Allsopp, 1952). The culture medium had also the same composition, but with various sugars replacing the 3 per cent. glucose of the original medium. As in the earlier work the cultures were kept in glass tubes

[Annals of Botany, N.S. Vol. XVII, No. 67, July, 1953.]

of 15×1·8 cm. containing 10 ml. of medium. The culture-room was maintained at 21±1° C. Light was supplied from a 'daylight' fluorescent tube (5 ft., 80 W.) which was automatically switched off for 12 hours in each 24-hour period.

Any comparative investigations were carried out on sporelings obtained by self-fertilization from one sporocarp. With occasional exceptions, for example albino sporelings, development was very uniform within the various batches and use of 6 or 8 sporelings per treatment was adequate for comparison of the effects of the different media.

Experimental Results

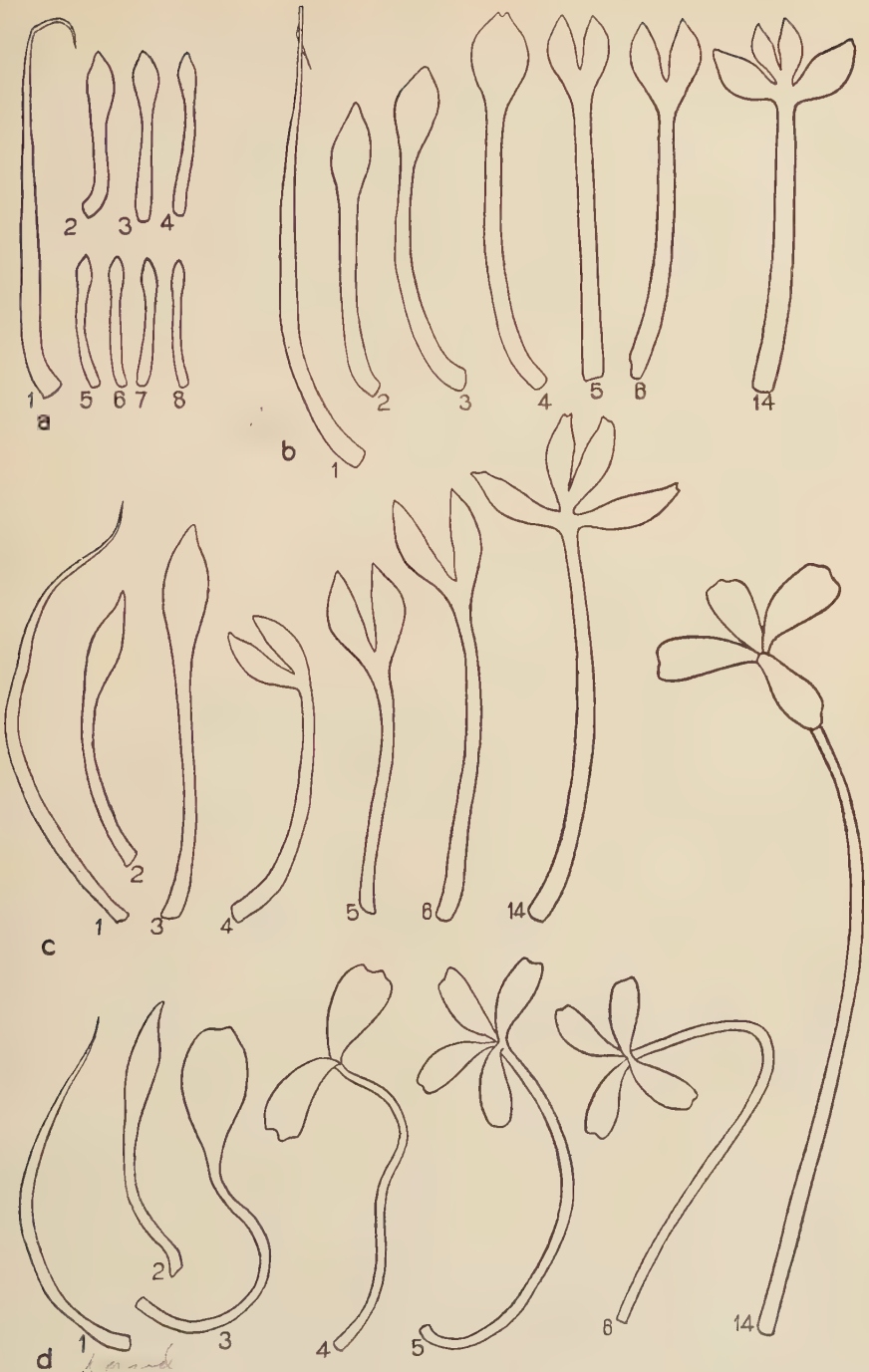
1. *The effects of sugar concentration*

An investigation using paper chromatography (Allsopp, 1951) had shown that the leaf of *Marsilea quadrifolia* contains considerable amounts of glucose, sucrose, and fructose, but that any other free sugars are present only in traces. The three sugars present in quantity were used therefore for the principal series of experiments.

(*a*) *Experiments with glucose.* Parallel sets of six cultures per concentration were set up using the basic medium without sugar and with 1, 2, 3, 5, or 10 per cent. glucose (B.D.H. Analytical Reagent).

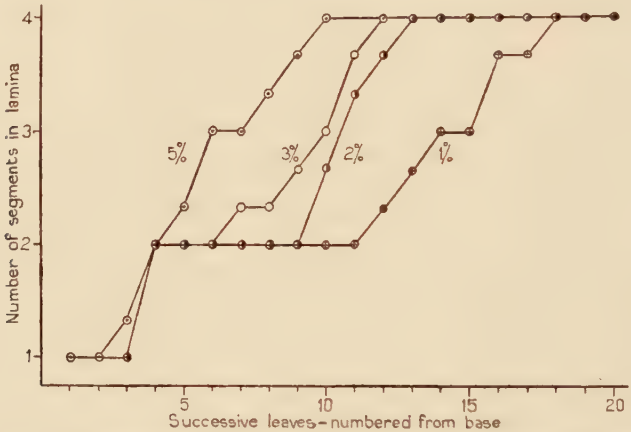
Sporeling development was markedly affected by the various sugar concentrations. Growth was very poor on 10 per cent. glucose, most of the plants dying within a few weeks after first producing several malformed leaves. The plants grew fairly well at all the other sugar concentrations. It was found that the glucose concentration has a distinct effect on the degree of division of the leaf lamina. Typical leaf successions from plants grown on the various sugar concentrations are illustrated in Text-fig. 1. As described in previous work (Allsopp, 1952) only simple leaves are produced when sugar is omitted from the medium. It is shown in the figure that at all sugar concentrations the acicular first leaf is followed by several spatulate leaves, which in turn are succeeded by one or more bifid leaves. The change from the simple to a distinctly bifid condition is usually abrupt, although one occasionally finds spatulate leaves with an apical notch (Text-fig. 1*b*, L4). Similarly there is usually a sharp change from the bifid to the quadrifid condition, trifid leaves being comparatively rare. In normal plants leaf division does not extend beyond the quadrifid stage, although more divided leaves are occasionally encountered in nature (Mahabale and Gorji, 1948).

The number of successive leaves required to complete the leaf sequence described is closely connected with the sugar concentration supplied. Text-fig. 2 records the mean values for the segmentation of the successive leaves of the six parallel series of cultures in the different glucose concentrations. It is shown that the final quadrifid condition is attained by earlier leaves in 5 per cent. glucose than in 1 per cent. The plants in 3 per cent. and 2 per cent. glucose occupy a middle position.



TEXT-FIG. 1. *M. Drummondii.* Successive leaves from sporelings grown on (*a*) basic inorganic medium only, (*b*) +1% glucose, (*c*) +3% glucose, (*d*) +5% glucose. (×6.) In Fig. 1*b*, L14 is the first quadrifid leaf. In Fig. 1*c*, L11 was the first quadrifid leaf; L14 is shown for comparison with 1*b*.

Morphological differences in the different glucose concentrations are not restricted to the degree of division of the leaves. Typical plants from each concentration after 10 weeks of growth are shown in Pl. XXV, Fig. 1. Apart from the differences in leaf segmentation already referred to and other relatively slight quantitative differences, it will be seen that the general appearance is similar in 1, 2, and 3 per cent. glucose but in the 5 per cent. sugar is quite distinct. The morphological differences are shown more clearly in Pl. XXV, Figs. 2 and 3, taken from a separate series of cultures (MD 5/10) which yielded almost identical results with the series (MD 4/10) discussed above.



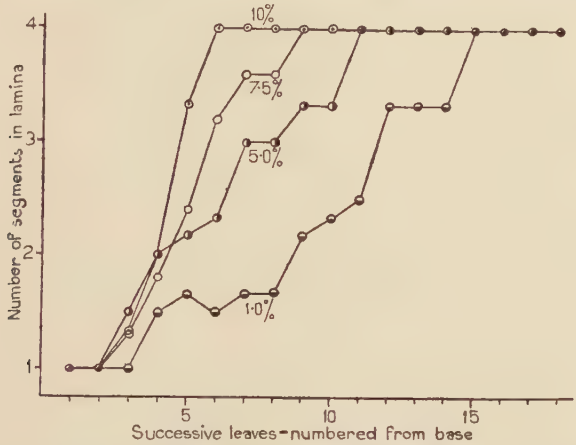
TEXT-FIG. 2. *M. Drummondii.* Effect of glucose concentration on the segmentation of successive leaves. Mean values from 6 parallel series of cultures. —⊕— 1% glucose; —◑— 2%; —○— 3%; —⊙— 5%.

The plant from 2 per cent. glucose has well-marked internodes in its rhizome, which bears thick, almost unbranched, roots and leaves with relatively small laminae but stout petioles. In contrast, the plant from 5 per cent. glucose has a short rhizome with crowded leaves and no obvious internodes. The roots are long, slender, and branched while the leaves have thin petioles and large laminae. The leaves show other differences (Text-fig. 1) which appear at the earliest stages of development of the sporelings.

In concentrations 1–3 per cent. glucose the lamina and petiole of the leaf lie in one plane, whereas in 5 per cent. glucose the divisions of the lamina are expanded in a plane almost at right angles to the petiole so that the quadrifoliate leaves are then apparently radially symmetrical. In 5 per cent. sugar solution the leaflets have a broad rounded outer margin in contrast with the pointed leaflets from the lower concentrations. The leaves from the lower sugar concentrations are readily wettable after exposure to air, but those from the 5 per cent. sugar solution have developed a smooth cuticle which is wettable only with difficulty.

The morphological differences described are similar to those encountered in nature in plants from different environments. The plants from 5 per cent.

glucose solution, although grown in a liquid medium, resemble the normal land-form, while those from the weaker sugar solutions are like the water-forms obtained by Glück (1911) from submerged plants of *M. hirsuta*. It is of interest that the balance between land- and water-forms is so sharp that intermediate types are not obtained. In 5 per cent. glucose solutions only land-forms develop and in 1 and 2 per cent. glucose only water-forms. In 3 per cent. glucose the cultures are mainly water-forms with an isolated land-form, while in 4 per cent. the cultures are land-forms with only an occasional water-form. Thus intermediate concentrations of sugar do not result in plants of an



TEXT-FIG. 3. *M. Drummondii*. Effect of sucrose concentration on the segmentation of successive leaves. Mean values from 6 parallel series of cultures. —◒— 1% sucrose; —◑— 5%; —○— 7·5%; —⊙— 10%.

intermediate type; the balance is tipped one way or the other and a varying proportion of land- and water-forms arises according to the particular concentration employed.

The results described above have been confirmed for a less extensive range of concentrations by culture series MD 8/5 and MD 18/12*b*, in each of which parallel sets of sporelings were grown on the basic medium with 2 and 4 per cent. of glucose respectively (Text-fig. 6).

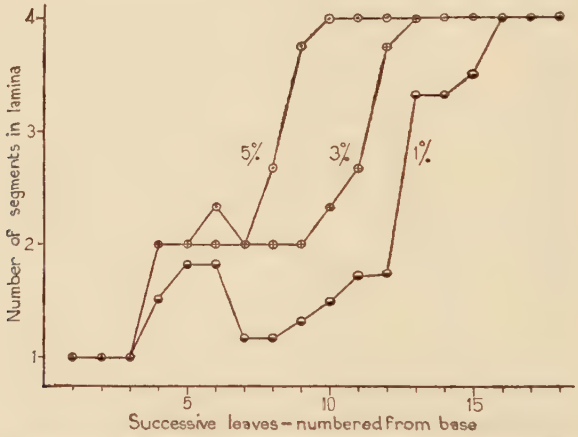
Essentially similar results were obtained from an experiment using *M. vestita* in which sporelings were grown on the basic medium with 1, 3, 5, or 7·5 per cent. glucose.

(*b*) *Experiments with sucrose. Series MD 16/1*: Parallel sets of six cultures per concentration were grown on the basic medium with 1, 3, 5, 7·5, 10, or 15 per cent. sucrose (B.D.H. Analytical Reagent).

In the 15 per cent. solution, development was slight and most embryos died off after a few weeks.

The effect of the sucrose concentration on the segmentation of the leaf was of a similar type to that already described for glucose, and is shown graphically in Text-fig. 3.

The general external morphology also showed changes corresponding to those described above for the various glucose concentrations (Pl. XXV, Figs. 4, 5, 6, 7, 8). The representative plant from 1 per cent. sucrose has the appearance of a typical water form while the plant from 5 per cent. sucrose shows the characteristic features of a land form. At 7·5 and 10 per cent. sucrose there is an increasing stunting of all parts and over the whole range of solutions employed there is a progressive shortening of the internodes with increasing sugar concentrations. It should also be mentioned that the highest concentration of sucrose at which growth is possible is almost double that of



TEXT-FIG. 4. *M. Drummondii.* Effect of fructose concentration on the segmentation of successive leaves. Mean values from 6 parallel series of cultures. –⊖– 1% fructose; –⊕– 3%; –⊙– 5 %.

glucose or fructose. The greater tolerance of the cultures towards high sucrose concentrations is probably connected with the lower osmotic pressure of sucrose solutions in comparison with glucose or fructose solutions of equal concentration.

(*c*) *Experiments with fructose. Series MD 16/1*: Parallel sets of six cultures were grown on the basic medium with 1, 3, 5, or 7·5 per cent. fructose (Kerfoot's Biochemical Reagent).

The 7·5 per cent. solution was evidently at the limit of tolerance and only 4 plants survived. These remained stunted like those in 7·5 per cent. glucose and 15 per cent. sucrose.

As shown in Text-fig. 4, the effect of fructose concentration on the degree of division of the leaf lamina is similar to that described already for sucrose and glucose. From the figure it will be seen that at the lowest concentration (1 per cent.) the degree of division of the lamina of successive leaves follows a somewhat irregular course. Such instability is frequently found at the lower sugar concentrations and probably indicates that until an extensive absorbing system is developed the amount of sugar entering the plant is scarcely sufficient to maintain the formation of any of the more divided types of leaf.

The effect of fructose concentration on general morphology is similar to that described above for the other sugars. Representative plants are shown in Pl. XXVI, Figs, 1, 2, 3, and 4. The plant from 1 per cent. fructose is a typical water-form and that from 5 per cent. a typical land-form. As in the sucrose cultures there is a gradual reduction in internode length over the range of sugar concentrations. The only marked specific effect of fructose is found in its somewhat injurious effect on the roots, which remain short and blackened.

2. *Comparison of effects of various sugars*

Although only sucrose, glucose, and fructose had been found free in quantity, it seemed desirable to study the availability of other sugars as a carbon source and to make a comparison of their morphological effects.

In *series MD 18/12a* parallel sets of cultures, all from the same sporocarp, were grown on the basic medium with 2 per cent. of each of the following sugars: the trisaccharide raffinose; the disaccharides, maltose, sucrose, cellobiose, and lactose; the hexoses glucose, fructose, mannose, galactose, and sorbose, and the pentoses arabinose and xylose.

A fairly good rate of growth was attained only by cultures on glucose, fructose, and sucrose. Growth was rather more rapid with glucose than with sucrose, but the final appearance of the plants from the two sugars was virtually identical.

The cultures in fructose were less well developed and the roots were stunted and blackened although the leaves were little affected. This response is the same as that described above for an earlier experiment although in the present experiment a different source of fructose was used (B.D.H. Laboratory Reagent).

The differences in the rate of growth of glucose and sucrose are paralleled by the effects of the sugars on the division of the lamina. From Table I it will be seen that a more divided leaf is attained rather earlier in the glucose than in the sucrose medium. With fructose there is an even greater delay. The more rapid growth on glucose than on sucrose and the earlier attainment of a more divided lamina in the former are confirmed for 2 and 4 per cent. concentrations in another series of cultures, MD 18/12*b*, described below. In *M. vestita*, also, a greater degree of segmentation of the lamina was attained earlier with 5 per cent. glucose than with 5 per cent. sucrose.

None of the other sugars investigated supported vigorous growth and some were distinctly toxic, permitting less growth than is found on the basic medium without any sugar. With mannose and galactose the embryo was arrested before the complete development of the first leaf, with sorbose and lactose only a second leaf was produced, while with arabinose 3 or 4 leaves appeared before death of the sporelings.

Of the remaining sugars best growth was obtained with cellobiose, followed by maltose. Only feeble sporelings developed in raffinose and xylose. After 6 months in the medium the cultures in raffinose had all perished, but slow growth was continuing in the other sugar solutions. The slight amount of

TABLE I

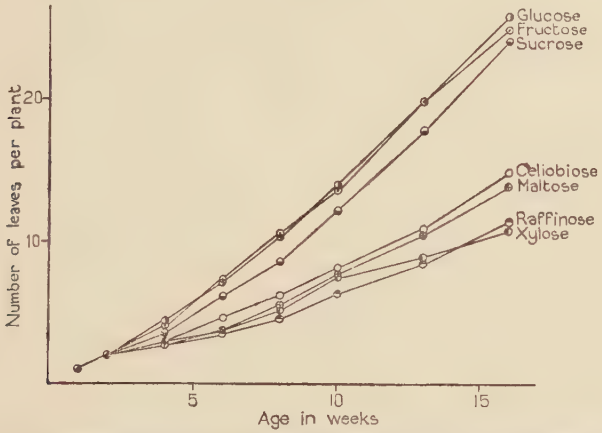
Effects of Different Sugars (at 2 per cent. concentration) on the Division of the Lamina of Successive Leaves (Marsilea Drummondii)

Number of Segments (Mean Values) in Successive Leaves

Sugar.	No. of cultures.	L3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Glucose	7	1·0	1·9	2·0	2·0	2·0	1·9	2·0	2·1	2·8	2·3	3·1	3·4	3·6	3·8	4·0	4·0
Sucrose	8	1·0	1·1	1·6	1·4	1·4	1·6	1·6	1·7	1·9	2·3	2·6	2·9	3·3	4·0	4·0	4·0
Fructose	8	1·0	1·6	1·5	1·6	1·3	1·1	1·4	1·4	1·4	1·9	1·9	2·1	2·4	2·6	2·9	3·2
Cellobiose	6	1·0	1·1	1·0	1·0	1·0	1·0	1·0	1·3	1·5	1·3	1·5	1·8	1·8	2·0	2·4	2·4
Maltose	10	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·1	1·1	1·3	1·3	1·3	1·4	1·8
Raffinose	10	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·1	1·1	1·0	1·0	—	—	—
Xylose	10	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	—	—	—	—

There was no growth on arabinose, galactose, mannose, sorbose, or lactose.

growth on these sugars is illustrated by Pl. XXVI, Figs. 5, 6, 7, 8, and 9, in which representative plants are compared with a glucose culture of the same age. From the figure it is also apparent that the plants from cellobiose, maltose, raffinose, and xylose have produced only simple acicular or spatulate leaves for a comparatively long period. During the period of the experiment (6 months) the xylose cultures bore only simple threadlike leaves. One plant had 20 leaves, all simple. In raffinose, most cultures produced only simple leaves, in this case usually with small pointed triangular laminae, but towards the end of the experiment two plants developed small bifid leaves. With



TEXT-FIG. 5. *M. Drummondii.* Effect of various sugars (at 2% concentration) on the rate of leaf formation. Mean values from 6–10 parallel series of cultures. –◑– glucose; –◒– sucrose; –⊙– fructose; –○– cellobiose; –⊕– maltose; –◐– xylose; –◓– raffinose.

maltose, most sporelings eventually attained the stage of bifid leaves, but even after 6 months only one plant had proceeded to the quadrifid condition. In cellobiose, division of the lamina was still retarded, but bifid leaves appeared earlier than in maltose, and by the end of the experiment all cultures had given rise to quadrifid leaves. Detailed figures for the various sugars are set out in Table I.

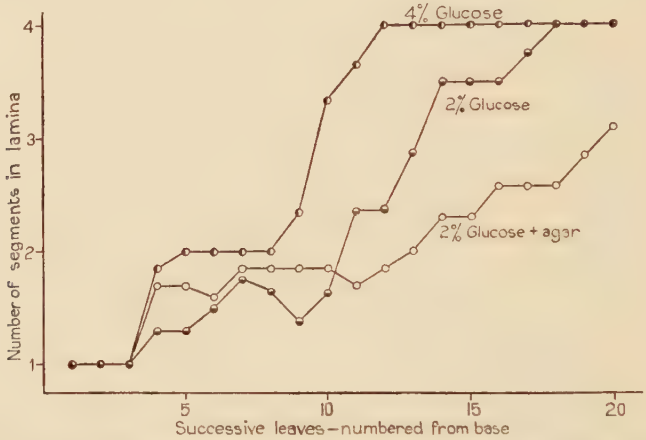
The rates of leaf formation on the various sugars are recorded in Text-fig. 5. These rates follow approximately the same order as the total growth-rates, but are relatively high in the sugars which support only feeble growth. Thus the rate of leaf formation in maltose is over half that in glucose, but the total growth is very much less, as is at once apparent from a consideration of Pl. XXVI, Figs. 5 and 7. It is also shown in Text-fig. 5 that in all the sugars the rate of leaf formation remains remarkably steady for a prolonged period. In this and other respects there is no indication of any adaptation of the type described by Spiegelman (1948) and others for certain micro-organisms.

The effect of sugar concentration on the rate of leaf formation was studied only for glucose, fructose, and sucrose. With glucose and fructose the optimum concentration was in the region of 2 per cent.; with sucrose at

approximately double that value. For the three sugars the different rates at the various concentrations remained approximately constant during the period of the experiment (4 months) apart from a slight falling off with time at the highest concentrations permitting reasonable growth.

3. *Comparison of development in liquid and on agar media*

In an earlier paper (Allsopp, 1952) reference was made to the poor growth on agar as compared with growth in liquid media, but no details were given of leaf development in the two types of culture. Studies of this kind have now been carried out.



TEXT-FIG. 6. *M. Drummondii*. Effect of medium on leaf segmentation. Mean values from 8 parallel series of cultures. —●— 2% glucose solution; —◐— 4% solution; —○— 2% glucose + 0·75% agar.

Series MD 18/12b. Parallel sets of cultures from the same sporocarp were grown on 2 per cent. glucose, 2 per cent. glucose + 0·75 per cent. agar, and 4 per cent. glucose; and on 2 per cent. sucrose, 2 per cent. sucrose + 0·75 per cent. agar, and 4 per cent. sucrose.

The effects of the various media on the leaf sequence are set out for glucose in Text-fig. 6. It is shown that growth on agar media delays the appearance of the final stage of division of the leaf. The results obtained with sucrose were similar, but with less marked differences between the cultures from the different media.

It is clear that growth on the agar medium reduces the vigour of the cultures. On glucose-agar, where the effect is more pronounced, the plants are very feeble as compared with those from the liquid medium. One of the better-developed cultures from the agar medium is illustrated in Pl. XXVI, Figs. 10 and 12. Cultures in liquid media were very similar to that shown in Pl. XXVI, Fig. 5, which is, however, from a different batch of cultures. With sucrose the difference in the amount of growth on the two media is smaller though still distinct. Plants from the two types of sucrose medium are illustrated in Pl. XXVI, Figs. 11 and 13.

Although the agar cultures showed a delay in the appearance of the quadrifid condition of the leaf, yet with both sugars, as shown in Pl. XXVI, Figs. 11 and 12, the radial land-form of leaf was finally produced. The helical roots appearing in glucose-agar (Pl. XXVI, Figs. 10, 12, and 14) have been considered in a previous note (Allsopp, 1952*c*).

Discussion

The view advanced by Goebel (1898) that the heteroblastic development of many plants is a consequence of a steadily increasing supply of nutrients to the growing-regions has been discussed already in earlier papers of the present series (Allsopp, 1952, 1952*a*). It was pointed out that this hypothesis has been supported by the demonstration by many workers, especially Goebel (1928), of reversion to juvenile leaves in plants grown under unfavourable nutritional conditions. Such reversion was obtained in aseptic cultures of *Marsilea* by restriction of either the organic or inorganic constituents of the culture medium (Allsopp, 1952*a*). There are also many examples in the literature of plants in which heteroblastic development is shortened in more robust individuals. All these observations point to the importance of nutrition in heteroblastic development, but so far as the present writer is aware, no worker has attempted to change the course of heteroblastic development by direct alteration of the carbohydrate supply of the plant. The experimental work described in the present paper was carried out with this object in view.

It has been found that in sporelings of *Marsilea* growing in aseptic culture there is a shortening of heteroblastic development with increase in the sugar concentration, fewer leaves of each intermediate type being produced before the final quadrifid condition is attained. The three sugars normally found in *Marsilea*, namely glucose, fructose, and sucrose, all gave a result of this type, but with slight differences between the individual sugars at any one concentration. Since the final quadrifid condition of the leaf was eventually attained in even the lowest sugar concentration (1 per cent.), the effects of the different media are to be referred to the concentration of the sugar rather than to any deficiency in total amount. Other experiments, which will be described in a different connexion in a subsequent contribution, have shown that when the osmotic concentration of the culture solution is increased, by adding mineral salts or a substance such as mannitol which is of no value as a nutrient, there is no indication of any abbreviation of the heteroblastic development. The effects of sugar concentration on the leaf sequence are therefore not of osmotic origin, but are probably to be explained by an increased rate of entry of the sugar into the plant. Street and McGregor (1952) arrived at the same conclusion as a result of their studies on the effect of sucrose concentration on the growth and morphology of tomato roots.

Additional support for the view that heteroblastic development has a nutritional basis is provided by a comparison of sporeling development on agar and in liquid cultures. With either sucrose or glucose, cultures on agar

were less vigorous than cultures in liquid media, and the stages of heteroblastic development were also appreciably extended.

The persistence of the juvenile stages in the agar cultures is clearly connected with the reduced growth on the agar medium, but the origin of the lower growth-rate is not so evident. It might be thought that in agar there is less diffusion, but this is scarcely a convincing explanation since the rhizome is continually extending into untouched regions of the agar. As another and perhaps more likely possibility, it may be suggested that in the agar cultures we are dealing only with absorption by the roots, whereas in liquid cultures the leaves also may play a prominent role.

With reference to the role of the leaf in absorption, it has been known since the last decades of the nineteenth century that when leaves are floated on sugar solutions they can take up the sugar to form starch (Boehm, 1883; Meyer, 1886). In 1916 Acqua and Jacobacci found that sugar absorbed by the cut surface of leaves of mulberry enabled growth to proceed more rapidly than in control plants without sugar. Knudson and Lindstrom (1919) were unable to obtain continued growth from albino corn plants when sugar was supplied to the roots, but Spoehr (1942) eventually achieved success by supplying sugar through the cut tips of the leaves. Went and Carter (1948) found that the roots of complete tomato plants take up very little sugar, but that it is absorbed readily by intact leaves, apparently through the whole surface and not especially through the stomata. Juhrén and Went (1949) were even successful in obtaining growth in darkness of plants of *Cucurbita pepo* by injecting sugar solutions into the hollow petioles.

But although these various works indicate that absorption of sugar proceeds more readily by way of the leaf than by the root, it has been known since the early work of Boehm (1883), Acton (1889), and Laurent (1898) that the roots of intact plants can undoubtedly take up sugar which is transported to the aerial parts. The present work with *Marsilea* has shown that this plant also can grow quite well when only the roots have access to the culture medium, although the amount of growth is less than in liquid media in which additional absorption by the leaves is possible.

Further evidence of the importance of nutrition is afforded by the results of the experiment in which a range of sugars was supplied to the cultures. In sugars such as xylose and raffinose, where growth was slight, only simple juvenile leaves were produced during the whole of the experimental period, while with maltose and cellobiose, in which growth was better although still much less than in glucose, fructose, or sucrose, the higher type of leaf was eventually attained, but only after long exposure.

In the experimental work described in the present and previous papers of the series (Allsopp, 1952, 1952*a*) it has thus been possible to extend, shorten, or reverse the stages of heteroblastic development in accordance with the nutritional conditions employed. There can consequently be no doubt as to the influence of nutrition, but its mode of action is less clearly defined. Troll (1939) has taken the view that heteroblastic development is dependent

on the size of the apical growing-point, which in turn is controlled by the supply of nutrients. In a recent paper Wardlaw (1952) has also emphasized the importance of the nutritional status of the sub-apical region. He had shown previously (1948) for certain leptosporangiate ferns that the shoot apex undergoes a considerable enlargement during the development of the individual plant.

The importance of the apex has also been stressed by Sinnott (1921), who concluded that the size of a plant organ is dependent, not upon the body size of the plant on which it is borne, but rather upon the size of the growing-point from which it developed. Following this suggestion, Whaley (1939) measured the volume of the apical meristem of several species of *Lycopersicon* at various stages of development and concluded that the volume of the apical meristem is correlated directly with the size of the determinate organs which it produces. He found (1939*a*) that as growth takes place the volume of the apical meristem increases, reaching a maximum late in the grand period of growth and falling off sharply to a relatively constant size. Working with *Zea mays*, Abbé, Randolph, and Einset (1941) found that the increase in relative width of leaf-blades 6–12 may be directly and quantitatively correlated with increase in size of the shoot apex from which the leaf initials arise.

From the literature cited it is clear that there is a progressive increase in the size of the apical growing-point during the early development of plants which have been investigated from this standpoint. Furthermore, in the varied experiments on *Marsilea* which have been outlined in the present series of papers, reversion to juvenile leaves was obtained only after plants had become attenuated with consequent diminution in the size of the growing-point. Small growing-points, as for example in the lateral branches arising from basal segments of the rhizome, always commenced growth by producing juvenile leaves, even under optimal conditions of culture (Allsopp, 1952*a*). It would therefore seem that the effects of nutrition on heteroblastic development are exerted via the growing-point. This view supplies an adequate explanation for the extension of the juvenile stages of development which in the work described in the present paper was achieved in three different ways; namely, by reduction of the sugar concentration, by use of a sugar permitting only feeble growth, or by growth on agar media. It is considered that in each case persistence of the juvenile stages was a result of failure of the apex to reach the minimum size necessary for the production of the more mature type of leaf.

Apart from the effect on heteroblastic development, a change in the sugar concentration had considerable influence on the general morphology of the cultures. Recent workers have made only incidental observations on morphological aspects, and it is to comparatively old works that one has to turn for any adequate account of the influence of organic nutrients on the morphology of plant cultures (Laurent, 1904; Molliard, 1907). From their extensive and thorough researches these workers discovered that, whatever the nature of the dissolved substance, in concentrated solutions there was always a decrease in length and a more or less marked thickening of the stem, although isotonic

solutions of different substances induced certain specific differences in the external morphology and anatomy of the cultures.

In the present work, cultures of *Marsilea* showed a progressive shortening of the axis with higher concentrations of each sugar tested. As in the work of Laurent (1904) it may be concluded that this shortening is induced by the osmotic concentration of the medium. This view is supported by a comparison of the effects of glucose and sucrose, and by experiments, not described in the present paper, in which mannitol was added to the sugar solutions. Similar effects of an increase in osmotic concentration have been described repeatedly, e.g. Beauverie (1901), Nightingale and Farnham (1936), Thimann and Schneider (1939), Hayward and Long (1941), Street and McGregor (1952).

Reference has already been made to the fact that at the higher sugar concentrations the entire aspect of the *Marsilea* plant is altered so that it has the appearance of the land-form rather than of the water-form found in the lower concentrations. These modifications in external morphology are accompanied by considerable differences in anatomical features, and will be considered in greater detail in a later contribution.

In addition to the effects produced by changes in concentration of an individual sugar, cultures of *Marsilea* showed characteristic responses to various sugars supplied at a uniform concentration. As in most other plants (summaries of literature in Miller, 1938; Lee, 1950*a*; and White, 1951) glucose and sucrose supported the best growth. The effects of the remaining sugars were similar to those described by other workers for an extensive range of plant materials. It should be mentioned, however, that different requirements have been found even in species of the same genus (Doerpinghaus, 1947) or in isolated organs of the same plant (Lee, 1950, 1950*a*). The toxicity of certain sugars towards *Marsilea* is paralleled by the findings for other plants (Knudson, 1915, 1916, 1917; Brannon, 1923; Steinberg, 1947; Street and Lowe, 1950), although Hildebrandt and Riker (1949, 1950) found that periwinkle callus tissue grows well on galactose, lactose, or raffinose, and other workers have claimed that certain plants grow better on fructose than on glucose or sucrose (Robbins, 1918; Brannon, 1923).

In conclusion, it may be said that where comparison can be made, the response of cultures of *Marsilea* towards different sugars or to various concentrations of an individual sugar is similar to that of most other plants previously investigated. It is therefore not unlikely that studies of the influence of nutrition on the heteroblastic development of flowering plants would yield similar results to those obtained from the pteridophyte materials used in the present work.

Summary

Sporelings of *Marsilea* were grown under aseptic conditions in liquid media containing various concentrations of glucose, sucrose, or fructose.

With each sugar it was found that the heteroblastic leaf development was curtailed with increase in the sugar concentration, fewer leaves of each inter-

mediate type being produced. This effect was not of osmotic origin, but was probably a consequence of increased rate of entry of the sugar into the plant.

Other morphological responses are described. At the higher sugar concentrations the sporelings, although growing in a liquid medium, possessed the features of the land-form of *Marsilea*, while in the lower sugar concentrations the features were those of the water-form.

A comparison was made of the effects of a range of sugars on the development and morphology of the sporelings. Best growth was obtained with sucrose or glucose. Fructose supported fairly good growth, but was somewhat toxic to the roots, which were stunted and blackened. Lactose, mannose, galactose, sorbose, and arabinose were definitely toxic and resulted in early death of the sporelings. Cellobiose, maltose, raffinose, and xylose were relatively poor nutrients but enabled some growth to take place. An account is given of the effect of the various sugars on heteroblastic leaf development.

The differences between the development of sporelings in liquid and on agar media are described. On agar media development was less vigorous than in liquid media and the heteroblastic development was correspondingly extended.

The experimental results are discussed with reference to related literature, and it is concluded that the heteroblastic development of many plants is determined by the effects of nutrition on conditions at the apical growing-point.

Acknowledgements

I am again indebted to Professor C. W. Wardlaw for his encouragement and helpful advice. I have also to thank Mr. E. Ashby for the photographs in Plates XXV and XXVI, with the exception of Pl. XXV, Fig. 1, which was taken by Mr. P. T. Dawes.

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DESCRIPTION OF PLATES

Illustrating A. Allsopp's article on 'Experimental and Analytical Studies of Pteridophytes, XXI. Investigations on *Marsilea*'.

PLATE XXV

Representative plants of *Marsilea Drummondii*

FIG. 1 *a*, *b*, *c*, *d*, and *e*. After 10 weeks of growth in media containing 0, 1, 2, 3, and 5% glucose respectively. (×2/3.)

FIGS. 2 and 3. After 18 weeks of growth in media containing 2 and 5% glucose respectively. (×1.)

FIGS. 4, 5, 6, 7, and 8. After 5 months of growth in media containing 1, 3, 5, 7·5, and 10% sucrose respectively. (×1.)

PLATE XXVI

Representative plants of *M. Drummondii*

FIGS. 1, 2, 3, and 4. After 5 months of growth in media containing 1, 3, 5, and 7·5% fructose respectively. (×1.)

FIGS. 5, 6, 7, 8, and 9. After 4 months of growth in media containing 2% glucose, cellobiose, maltose, raffinose, and xylose respectively. (×1.)

FIG. 10. From medium containing 0·75% agar and 2% glucose, after 14 weeks of growth. (×2.)

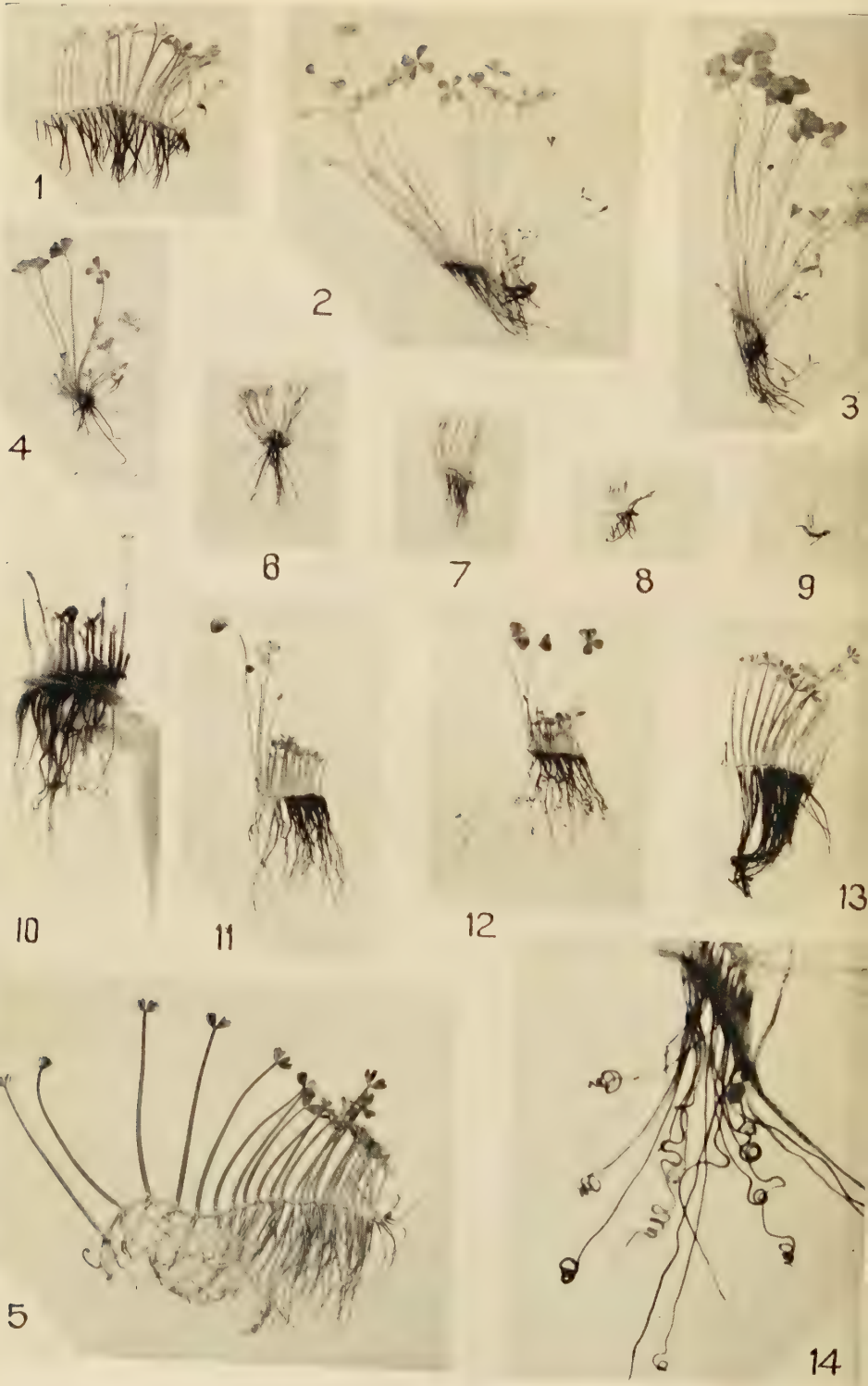
FIG. 11. From medium containing 0·75% agar and 2% sucrose, after 17 weeks of growth. (×1.)

FIG. 12. From medium containing 0·75% agar and 2% glucose, after 17 weeks of growth. (×1.)

FIG. 13. From liquid medium containing 2% sucrose, after 17 weeks of growth. (×1.)

FIG. 14. Root system from medium containing 0·75% agar and 2% glucose, after 17 weeks of growth. (×4.)

A. ALLSOPP



A. ALLSOPP

Gynospore or Megaspore—A Restatement

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ABSTRACT

The current use of the terms 'megaspore' and 'microspore' with reference to the biocycle of seed plants implies that seeds, in fact and of necessity, arose from sources having typical heterospory. Measurement of spore size in gymnosperms shows that the spores are at least sub-equal and that in conifers the pollen spore is more frequently the larger. *Sequoia sempervirens* is an example of a living plant which shows, in early development, a direct transition to the seed condition from what is essentially a homosporous condition. Seed origin from homosporous sources cannot, on the present evidence, be ruled out. Pending decisive evidence non-committal terms should be used. Gynospore and androspore are suggested as suitable, being simply descriptive of the respective spore function.

THE writer in his work on conifer embryogeny has consistently used the terms 'gynospore' instead of 'megaspore' (or 'macrospore') and, where necessary, 'androspore' instead of 'microspore'. This note purports to give, briefly, a restatement on that usage. Though the use of the actual term 'gynospore' may be personal, no originality is claimed for the concept behind it, which derives from the paper published by R. B. Thomson in 1927.

The use of the commonly accepted terms 'microspore' and 'megaspore' to designate the spores of seed plants is firmly entrenched in botanical writing and teaching. The young student is introduced to the comparison of the biocycles of *Selaginella* and the seed plant, a useful and necessary discipline. But the comparison is pushed farther and the student is taught, at least implicitly, that the condition in seed plants is one of modified heterospory, that the seed is a derivative of a heterosporic stage. Unless one proposes to give quite different meanings to the same term then heterospory, in this connexion, is the heterospory displayed in, say, the lycopods or water ferns.

Such a claim is a bland assumption. When exactly, and by whom, this concept was first clearly expressed in its present form is unknown to the writer. It is sometimes fathered on Hofmeister, but Thomson is correct when, in his examination of Hofmeister's actual words, he reiterates that the concept, as at present expressed, is not to be found in the writings of Hofmeister, who limits himself to essential comparisons.

It is not intended here to deny that seeds may have been derived from heterosporous sources, but even a short objective examination shows that one

[Annals of Botany, N.S. Vol. XVII, No. 67, July, 1953.]

cannot exclude the equal possibility of an origin from a homosporous condition. The point is that the continued use of the terms 'microspore' and 'megaspore' formally commits us to the acceptance of one assumption to the exclusion of the other; while the terms 'gynospore' and 'androspore' are non-committal and permit an open approach to be kept to the problem of seed origin until more is known about the course of that evolution.

The general fossil evidence hardly helps us to a decision. The seed appears widespread in the Carboniferous with no real clue to its origin. In the cordaitalean-conifer complex one can go back to *Callixylon* and, assuming with some degree of probability the seed nature of this plant, can say no more than that the seed must have taken its origin in this line earlier than the Upper Devonian. The position is no clearer in the pteridosperm line. The occurrence of true heterospory in *Archaeopteris latifolia*, and perhaps in other species of the genus, may be cited, but there is no proof that this occurrence was connected with seed evolution. It may have been but, as it stands, we simply have the record, even if fully confirmed, of the occurrence in Upper Devonian time of heterospory in a plant of somewhat uncertain relationships.

Evidence might then be sought among living plants. If heterospory, such as is shown in *Selaginella*, is basal to seed formation one might expect to find traces of the condition among the spores of living seed plants. One might expect some evidence that the micro- (or small) spores were small and the mega- (or large) spores were large. It was Thomson (1927) who first examined this question of comparative spore size in gymnosperms and angiosperms. From the data available he claimed that the pollen spore and seed spore were, on the average, equal in size, with, in some cases, the pollen spore the larger, in others the seed spore. He claimed that there was no cogent factual evidence in seed plants that the spore condition was heterosporic. The present note records further observations on this point.

Criteria Employed

Thomson unfortunately was almost entirely dependent for his records on measurements from published drawings by various authors. In some cases the most suitable stages for measurement were not figured; and in all he was dependent on the accuracy of the magnification value recorded by each worker consulted. Thus the writer, though freely accepting Thomson's claim at the time, has long felt that the matter deserved a fuller examination and particularly that evidence from directly comparable measurements was desirable.

Before, however, undertaking direct measurements it was necessary to define the actual stages to be measured and recorded. It was clearly desirable to measure the size of *spores* and not of *young gametophytes*. The pollen of the larch, for instance, which at shedding has two prothallial cells as well as stalk and body and tube nuclei, was considered a young gametophyte. The standard for pollen-spore measurements seemed fixed by reference to forms such as *Fitzroya* and *Juniperus* in which the pollen is shed in the uninucleate

condition. Here the pollen is a true, free, uninucleate spore. In these cases, however, the pollen when liberated from the tetrad is, of course, still young and with a relatively thin wall, but it increases in size a little as it rounds off and as the wall strengthens. So, in general, the stage measured was as close as possible to the condition in which after liberation from the tetrad the grains had well rounded off and had matured their wall but before the prophase of the first division. In fact, in most cases, especially in cupressoid and taxodioid pollen shed in the binucleate condition, very little relative error is introduced by taking the size of the mature grain at shedding. Only in a few cases, of which *Larix* is one, does any appreciable increase in size take place between the uninucleate stage and shedding.

The seed spore presented slightly greater difficulty. Judging from the ease with which ovular tetrads can be found when the approximate date of their occurrence is known it seems possible, in conifers at least, that the tetrad, including the functioning spore, rests for some time before germination. This functional spore in the tetrad might be legitimately considered mature, but it was felt that a measuring of the spore at this stage might be weighing the balance against it in a size comparison. On the other hand, the first stage in spore development in conifers is an expansion with the development of a large central vacuole surrounded by a thin protoplasmic layer in which the nucleus lies. *It is here taken that the first vacuolation is the beginning of spore germination.* In cases where a fairly complete series is available it is clear that the whole ovule is developing at this stage. The ovular cells are in division, nucellus and integument are extending, pollination has been effected, and the pollen has germinated. Once vacuolation has set in development continues and follows its determined course to fertilization. During at least the earlier stages of development two phases appear—stages of expansion, with nuclei in interphase, separate each of the stages of more or less simultaneous nuclear division. It would appear then that the first stage in development is an expansion and vacuolation, followed by the first division, then further expansion to the second division, and so on. Unless the first vacuolation of the functional cell in the tetrad be taken as the early germination of the spore, then the 'spore' condition must be visualized as covering the whole early 'prothallus' up to alveolar and primary cell formation. This rather metaphysical position is not here being argued; for the purposes of this note it is not accepted.

It was decided therefore to measure the spore at a stage when germination had well begun, i.e. as near as possible to a stage when vacuolation was clearly already quite distinct and when the non-functioning spores were in an advanced condition of degeneration or completely collapsed.

Data Records

As Thomson (1927) gives a very comprehensive list of literature it seemed unnecessary to repeat it here, and so in the literature list appended to this note only papers not referred to by him are included. Authors referred to in

the text and not here cited are readily traced by reference to Thomson's list.

Likewise in the tables of spore size, those records with an asterisk have been reassessed from papers also readily identified by reference to Thomson's tables. Measurements taken from more recent papers are indicated by a number in brackets *before* the plant name, the number referring to the literature reference number here appended. The other records are new.

The results are tabulated as broad ratios, partly because in such a form the relations are more diagrammatic, but also because no great accuracy can be claimed for them. Thomson, by recording values to the first decimal place, was clearly claiming much greater accuracy than could be justified. Apart from the obvious fact that no two successive ovules would show gynospores of exactly the same size, the gynospore is often of no definite geometrical shape, sometimes indeed rather irregular. No matter how carefully attempted the measurements at best can be only reasonable approximations. It is not claimed, for example, that a recorded 1·5:1 ratio might not come out in another set of ovules as 1·4:1. Indeed in cases closer to equality, as 1·2:1, another observer, with whom the gynospore stage was a little more advanced, might record a 1:1·2 ratio, yet obviously without disturbing the idea of the subequality of the spores. But great changes would not arise no matter how wide a range of material was available. A change of ratio from, say, 2:1 to 1:2 is probably out of the question. Taking pollen size as reasonably constant, which it is, such a ratio change would mean a fourfold difference in the estimated gynospore size—from, say, 7,000 μ^3 to 28,000 μ^3. If the germinating gynospore measured as 7,000, on the standard here used, was even reasonably suitably selected then, without question, a spore stage measuring 28,000 would be so advanced in appearance and vacuolation that it would be straight away rejected. Even an increase in gynospore size sufficient to change a 1·5:1 ratio to a 1:1·5 ratio would bring the gynospore well outside the normally accepted range of development. Averages of large numbers of readings might be thought desirable as giving greater accuracy, but it is clear that such greater accuracy would not affect in any material way the *broad* nature of the results.

As a preliminary it is useful for comparison to put down the ratios in a few typical cases of true heterospory:

TABLE I

Microspore–Megaspore Ratios in Heterosporous Types

Selaginella scandens	1:30,000	*Marsilia quadrifolia*	1:800
S. elegantissima	1:230	*Lepidostrobus* sp.	1:1,100
S. (average of 8 sp.)	1:1,500	(1) *Archaeopteris latifolia*	1:1,000

Vol. range of megaspore: $7 \times 10^6 \mu^3$ to $350 \times 10^6 \mu^3$.

For conifers, the androspore–gynospore ratio, A:G, and the approximate size of the gynospore, as shown in Table II, are in striking contrast.

TABLE II

Androspore–Gynospore Ratios (A:G) for Conifers

	Plant.	A:G.	Vol. G. in cu. μ.
	Podocarpus andinus	2:1	8,200
	,, *nivalis*	1:1	4,250
	Saxegothaea conspicua	3:1	5,200
	Taxus baccata	1·4:1	10,200
	,, *canadensis**	1·2:1	3,500
(5)	*Torreya californica*	1·2:1	8,000
	Agathis australis	1·7:1	20,300
	Araucaria araucana	4:1	17,500
		2·5:1	
	Pinus silvestris	2·5:1	4,020
	,, *laricio*	2·7:1	8,200
	Larix decidua	2:1	25,000
	,, *leptolepis*	3:1	13,000
	Cryptomeria japonica	2·6:1	5,700
	Sciadopitys verticillata	1·2:1	5,000
	Sequoia gigantea	1·5:1	6,000
	,, *sempervirens*	2:1	5,500
	*Cunninghamia sinensis**	1:1	10,100
	Athrotaxis selaginoides	1·8:1	6,500
(2)	*Taxodium distichum*	1:2	5,900
	Callitris sp.	1:1	2,500
(6)	*Actinostrobus pyramidalis*	2·5:1	4,000
(9)	*Thujopsis dolabrata*	2:1	3,500
	*Juniperus communis**	1:7·5	5,100

* Data reassessed from Thomson references.

While further notes could be given about nearly all these records, only certain additional comments are strictly necessary.

In *Larix leptolepis*, for instance, there was no gynospore stage available between a fairly mature tetrad yielding a 4:1 ratio and an advanced vacuolate stage. Data, however, from *L. decidua* showed that a rather advanced vacuolate stage was about 50 per cent. greater in volume than a youngish tetrad, a figure of the same order as in other genera. The actual measured tetrad in *L. leptolepis* was therefore stepped up 40 per cent., giving the recorded 3:1 ratio. An occasional similar minor adjustment was made elsewhere, but always, as here, in favour of the gynospore.

In some cases, however, there is a very marked discrepancy between ratios derived from Thomson's figures and the ratios here recorded. Thus Thomson's A:G ratio for *Sciadopitys* is 1:5, whereas here the spores are recorded as sub-equal. Reference to the figure in Lawson's account of *Sciadopitys*, upon which Thomson depended for his measurement of the uninucleate gynospore, shows it to be a very advanced vacuolate stage just ready for nuclear division and so inadmissible on the standard here used. A wide range from young tetrads to vacuolate stages was available for the record here given which is actually the average of a number of measurements from different ovules. The same conditions, but in an even more exaggerated form, account for the discrepancy between Thomson's ratio of 1:7 for *Araucaria brasiliensis* and the ratio here of 4:1 (or 2:5:1) for *A. araucana*. The fact, too, that in Thomson's

records for *A. brasiliensis* the binucleate pollen grain is only about one-half the recorded volume of the uninucleate grains suggests that none of his figures pertaining to this species need be taken too seriously. In *A. araucana* it is thought that the 4:1 ratio is truer than the 2·5:1. Influenced perhaps by the unacceptable 1:7 ratio of the other species and by the more concrete fact that in the araucarians a much more rapid expansion and vacuolation takes place on gynospore germination than is common elsewhere, a number of measurements were averaged including some stages so advanced in vacuolation that they would have been at once rejected in other types. The 2·5:1 ratio so arrived at only emphasizes the greater size of the androspore in this plant. The much smaller ratio in *Agathis* is largely accounted for by the fact that the pollen is much smaller—a point also recorded by Thomson.

There are thus only two records in which the gynospore is noticeably larger than androspore, viz. *Taxodium distichum* and *Juniperus communis*. Unfortunately fertile material of neither was available for personal examination. The *Taxodium* ratio is from figures by Coker (1903), and unless some serious error arose in the magnifications given by him the record stands. It will be noticed, however, that the 1:2 ratio does not arise because the gynospore is unusually large, its size—5,900 μ^3—being about the average, but as a result of the relatively smaller size of the pollen. This change in the ratio in favour of the gynospore due to the small size of the pollen is very striking in the case of *Juniperus*. Assuming the correctness of the magnification values given by Noren and Ottley the gynospore is even a little less than average, about 5,000 μ^3, but the pollen grain is only about 650 μ^3, which is very small indeed for coniferous pollen. There does exist incidentally some evidence such as this to the effect that members of certain different conifer lines show as a common feature a tendency to reduction in pollen size. *Agathis* may be another case in point. In connexion with *Juniperus* it may be further worth mentioning, as an example of the necessity of checking all Thomson's numbers, that he gives two records for the binucleate gametophyte, one from Ottley of about 3,500 μ^3 and one of 20,000 μ^3 from Noren, though references to the pertinent figures shows that practically the same stage has been drawn in both cases. The explanation of the discrepancy is simple. The Ottley figure is one of a strongly plasmolysed gametophyte lying well within the much larger cavity which it originally occupied. Thomson measured only the protoplasmic limits of the contracted stage.

On the standards here adopted, there is thus no sign of morphological heterospory in conifers. The material available, though far from comprehensive, covers all the families, and there is no reason to expect that the relations would be altered even if a more complete survey were possible. Such a survey might yield other results. It might show relations between spore size and certain generic groupings, or it might show tendencies to enlargement or reduction of spore size in certain lines, and the like, but the general results would surely stand. The spores may be subequal; the androspore, with surprising frequency, may be larger, even much larger, than the gynospore;

and in the few cases where the gynospore is definitely the larger this seems due to a special size reduction in the pollen.

The cycads and *Ginkgo*, with ovules almost Palaeozoic in type, might be expected to show indications of morphological heterospory if such still survived in living plants, but such is not the case. Records, however, of suitable stages of these are surprisingly few. The principal ones available, including also the specialized and possibly relatively modern *Ephedra* and *Welwitschia*, are here summarized. The reference symbols are as in the previous table but the data, rechecked and modified from Thomson, are included here as they deserve a short explanatory statement:

TABLE III

Androspore–Gynospore Ratios (A:G) for Gymnosperms other than Conifers

		A:G.	Vol. G. in cu. μ.
	*Ginkgo biloba**	1·3:1	7,300
		1:1·6	
	*Ceratozamia longifolia**	1·5:1	17,500
		1:1·5	
	*Stangeria paradoxa**	1:2·8 (?)	14,000
	*Zamia floridana**	6·5:1 (?)	900
		2:1	2,200
	*Ephedra trifurca**	1:2	2,450
(4)	,, *foliata*	1:1·2	2,250
	*Welwitschia**	3:1	4,500

* Data reassessed from Thomson's references.

In this list the cycads and *Ginkgo* definitely need revision. The spore size measurements are derived from oldish records, some quite old, as Juranyi's notes on the pollen of *Ceratozamia* which date from 1872. In many cases the figures are poor and the magnifications doubtful, so that, from the data available, different ratios may readily be derived for the same type. Thus in the case of *Ginkgo* there is only one gynospore drawing available from Carrothers at approximately the right stage. It is perhaps a trifle young, but without the other stages of development for comparison it could be accepted as a rough estimate. But the pollen sizes differ enormously in the records. Strasburger's uninucleate stage measures about 9,500 μ^3, his 2-celled stage 11,000 μ^3, and his 3-celled stage something over 12,000 μ^3; while Hirase's recorded 3-celled stage only measures 4,500 μ^3. It may be that there are special horticultural races, in which case the spores must be measured in specimens of the same race. A survey of the figures published by Juranyi and Treub for *Ceratozamia* shows similar as well as other difficulties. The correct ratio probably lies in each case between the two approximate ones given in the table, the spores being probably sub-equal. The ratio for *Stangeria* should be closer to equality as the only available figure of the developing gynospore, quite a poor one, is in an advanced stage of vacuolation, probably approaching the first division. The gynospore, if any analogy holds between cycad and conifer development rates and stages, can only be a little, if at all, larger than the androspore.

On the other hand, the condition in *Zamia* seems unusual. The androspore is definitely larger than the gynospore, probably much larger, though the 6·5 : 1 ratio may be too high. Thus pollen size is again uncertain. Smith gives two figures of mature pollen both just beginning to germinate. One, on the nucellus of an ovule, measures 3,600 μ^3, the other, developing in culture, 6,000 μ^3. Thomson gives 5,400 μ^3 as the size of mature shedding grains actually measured by him and 6,000 μ^3 for grains at the uninucleate stage, again measured by him from sections prepared, but not drawn, by Smith. From a comparison with published accounts of pollen development in other cycads it would appear that some increase, but no great increase, takes place in maturing from the uninucleate stage. If 5,400 μ^3 be a correct average for mature grains directly measured, then the uninucleate stage, despite the records from Smith's sections, might be rather of the order of 4,500 μ^3. Smith figures stages in gynospore development, viz. a late tetrad with vacuolation apparently well begun, a very advanced vacuolate condition, and a binucleate stage just after the first division. The sizes are, respectively, 900 μ^3 and 3,600 μ^3—the two latter stages being roughly equal. The late tetrad, at a stage apparently quite up to the standard here used, is extraordinarily small for any gymnosperm and unexpectedly so in a cycad. It is this 900 μ^3-stage which, with the 6,000 μ^3-androspore, yields the 6·5 : 1 ratio or, with the 4,500 μ^3-measurement, a 5 : 1 ratio. The 3,600 μ^3-gynospore stage is definitely far in advance of anything even reasonably acceptable on present standards; and, even by averaging the 900 μ^3- and 3,600 μ^3-stages, a measurement still recognizably too high for the gynospore, and coupling it with the 4,500 μ^3-androspore, the A : G ratio can only be cut to 2 : 1. The androspore here must be distinctly the larger of the two. There is no reason for doubting the greater size of the androspore in *Welwitschia*, though revision might show the 3 : 1 ratio to be a little high.

It is therefore suggested that careful revision of the spores in these gymnosperms, if examined formally for spore size, would show a pattern strictly comparable to that in conifers—spores sub-equal, the androspore the larger, and perhaps occasionally, the gynospore the larger. The shifting of the ratio in favour of the gynospore due to small pollen size appears here also, as shown by *Ephedra*. The gynospore volumes are of the same order as those in conifers.

No proper study has yet been made in angiosperms along these lines. Thomson gives a dozen records culled from the literature. These have not been checked, but the general pattern is again the same.

Reference has been made to the increase in size of the gynospore on germination. The ratios between binucleate pollen (A2) and binucleate gametophytes (G2) at various stages of development are tabulated in Table IV for some authenticated cases. A number of other records might have been included, but as these, though probably satisfactory, involved some small guess-work in one direction or another they were omitted. The selection in Table IV is reasonably representative and is included as a matter of interest,

since comparison with Table II gives easily an idea of the relative increase in size of the gynospore in those species common to both tables. For this purpose the size of A1 and A2 may be taken as roughly equal. Only uninucleate pollen is given for *Actinostrobus* and *Austrotaxus* as the binucleate condition is not attained till the grain germinates on the nucellus, and no measurable figures are available.

TABLE IV

Ratios of Binucleate Pollen and Binucleate Prothalli (A2:G2) in Gymnosperms

		A(2):G(2).	Stage of development of G.
	Athrotaxis selaginoides	1·5:1	Telophase of 1st division
(6)	*Actinostrobus pyramidalis* (A1)	1:1·7	After 1st division
	Larix decidua	1:1·2	,, ,, ,,
	*Zamia floridana**	1·6:1	,, ,, ,,
(7)	*Austrotaxus spicata* (A1)	1:1·7	Developing
	Sequoia gigantea	1:2	,,
(4)	*Ephedra foliata*	1:4	,,
	Athrotaxis selaginoides	1:5	,,
	Pinus silvestris	1:11	Well advanced
(5)	*Torreya californica*	1:13	,, ,,
	*Cunninghamia sinensis**	1:14	,, ,,
	*Ephedra trifurca**	1:15	,, ,,
	*Araucaria brasiliensis**	1:24	Near 2nd division

* Data reassessed from Thomson's references.

The table shows that, even using well-advanced binucleate stages incorporating the second phase of expansion, the ratios do not yet even approach those shown in cases of true heterospory.

DISCUSSION

Another aspect deserves consideration. In spite of the uncertainty of the position, it has been long maintained not only that seeds did in fact arise from a heterosporous condition but that such a condition was a *necessary* antecedent to the seed. Arnold (1947), to take but one example, commenting on heterspory in *Archaeopteris* says that this plant 'may constitute the necessary heterosporous stage in the derivation of the seed from the terminal sporangium of the Psilophytales'. The idea of such *necessity* is, of course, just another bland assumption, but so much speculation has been expended on the idea, and hypothetical stages of the progression from heterospory to the seed have been so often worked out, that the fact that the idea is an assumption has become rather obscured. A common position would seem to be that suitable intermediate stages *must* be somewhere in the fossil record simply awaiting discovery. If, however, it were once accepted, even provisionally, that a homosporous origin is possible and if equal energy be once devoted to working out the possible progression stages from a homosporous ancestry, then the position becomes fluid and subsequent evidence, as it accumulates, can be examined on its merits and without bias.

Early development in *Sequoia sempervirens* seems to bear directly on the

question. It is in no way maintained that *Sequoia* proves that seeds arose from homosporous sources since *S. sempervirens* is a derivative polyploid, probably a hexaploid according to Stebbins (1948), but it is an example in a living plant of the direct passage from homosporous conditions to seed conditions. Reference to Table II shows that in this form the androspore is actually the larger spore.

The young ovule of *S. sempervirens*, described by Looby and Doyle (1942), develops many spore mother-cells and a definite tapetal layer. Each mother-cell separately forms a tetrad so that at this stage a T.S. shows a striking similarity to a developing pollen-sac except perhaps in the obviously more physiologically active outer layers. The tetrads show grades between linear tetrads, with one large lower cell, to tetrahedral tetrads with four equal spores. At least one spore from each tetrad germinates, so that a plexus of young gametophytes are formed growing basipetally and, in shape, like broad pollen-tubes within which the first free nuclei are formed. Commonly one becomes established to give the functioning prothallus, though, as is to be expected from the conditions of origin, supernumerary prothalli are more frequent than usual in this species.

We have here a direct transition in no way related to morphological heterospory but related rather to dynamic physiological change. The primary phenomena is the changed function of the sporangium, the nutrition of retained spores and young gametophytes; and, since such a transition is an observed fact in a living plant, could not some such direct change have occurred in an early pteridophyte? Indeed a direct physiological change of this type is of so simple and direct a nature that one might expect it to have occurred more than once in the past. No long series of hypothetical progression changes need be imagined or sought for, and the absence from the fossil record of suitable intermediate stages between heterospory and the seed may be simply accounted for by the consideration that they were never there. Apart from the integument the ovule of *S. sempervirens* cannot greatly differ, and then only in degree but not in kind, from a young developing sporangial tip of a psilophytalean form. In the conifers, from such a basal idealized type, with the primary physiological change referred to impressed upon it, there is little more than a reduction in number of the functioning spore mother-cells. Many conifers still develop a number of mother-cells of which commonly only one, not particularly different from the others, develops to a tetrad. Most, however, have advanced of course to the condition in which only one mother-cell develops a tetrad, with one resulting functioning spore, though in some the tapetal tissue present may have a sporogenous origin.

Conclusion

It is not the purpose of this note to speculate further on seed problems. The difficulties presented are not being overlooked; essentially the difficulties inspired the note. It is again emphasized that the possibility of seed origin from heterosporous sources is in no way denied, but direct evidence for such

has been sought for in gymnosperms and not found. Such evidence as has been presented, from relative spore size and from analogy with development in *Sequoia sempervirens*, not only does not preclude a homosporous origin but, in fact, seems strongly to suggest that the idea of such an origin may merit fuller consideration than it has been given.

Such consideration may profoundly affect the approach to seed problems. Concepts of static morphology may be found to yield to concepts of dynamic physiological activity. The condition of true morphological heterospory may appear a highly specialized one, not sufficiently plastic, and the fact that it produced *Lepidocarpon* and *Miadesmia* may even be taken as an indication that it could produce nothing else. The idea of homosporous origin, somewhat along the lines actually shown by *Sequoia sempervirens*, throws the weight not on spore size but on the physiological activity of the sporangia; and the ovule, from its first rudiment, is the seat of a whole series of predetermined and connected dynamic changes. Alveolar and cell formation, fertilization, embryogeny—these are all active dynamic processes determined in sequence, though we know nothing of the nature of the determination or of the metabolic actions characteristic of each phase. So, for example, if a cellular gametophyte in a conifer is bounded by a somewhat cutinized membrane it need not be interpreted as a spore coat inherited from the heterosporous ancestor, a sort of vestigial morphological structure. During development in the ovule there are the two tissues, one haploid the other diploid, each certainly dominated by intensely different metabolism, and these are separated by a membrane. From the point of view of dynamic physiology it is hard to conceive a spore membrane, with no primary function, developing and thickening with age—it seems more probable that it has a function or it would not be there. Again, the homosporous approach avoids the difficulty of considering as the spore all the developments from the tetrad up to the initiation of alveoli or the formation of primary walls. Unless the immediate post-tetrad stage be considered as the early germination of the spore, the whole free nuclear stage, up to at least the alveoli, must be considered as the spore. That may be a legitimate interpretation, though it is difficult, for one familiar with development stages in many types, to include such stages in some of them, notably again *Sequoia sempervirens*, under the concept of 'spore'. The extension, *a priori*, of the spore concept to include these stages may be merely a *petitio principii*; it may be a straining of the concept in order to avoid a dilemma, viz. *A* is equal to or greater than *B* but still *A* must be called very small and *B* very big!

At the risk of tedium it is again stated that no claim is here made for a homosporous in preference to a heterosporous origin. It is claimed, however, that a homosporous origin is equally possible and cannot be ruled out. The continued use of the terms 'microspore' and 'megaspore', the continued adherence, with no real confirmatory evidence, to an assumption of heterosporic origin involving the exclusion of any other assumption at least ignores difficulties and seems illogical and unscientific. In the present conditions of

uncertainty, it seems more reasonable to use terms which do not imply either view and which keep the approach open for an unbiased reception of new evidence. The terms 'gynospore' and 'androspore' are simply descriptive—the spores giving rise respectively to the female and male gametophytes. Better terms might be suggested. Thomson's own first suggestions were 'angiospore' and 'doulospore', but these seemed more awkward and less apt than 'gynospore' and 'androspore'. The terms, however, do not matter as long as they do not imply an unproved assumption.

Summary

The use of the terms 'microspore' and 'megaspore' in seed plants implies the acceptance of the idea that seeds were derived from heterosporous sources. This is an assumption. In this paper the relative sizes of pollen and seed spores in many gymnosperms, principally conifers, are listed. The spores are frequently sub-equal but more often the pollen spore is the larger. Where the seed spore is markedly the larger this seems to be due not to any enhanced size of it but to special reduction in size of the pollen. There is thus no evidence of morphological heterospory. *Sequoia sempervirens* is quoted as an example of a direct transition from homosporous conditions to the seed. Seed origin from homosporous sources seems therefore possible and cannot be ruled out. Terms therefore that do not commit to either view should be used instead of megaspore and microspore pending decisive evidence. The terms 'gynospore' and 'androspore' are such non-committal terms, being simply descriptive of the spores giving respectively the female and male gametophytes.

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The Initiation, Growth, and Emergence of Leaf Primordia in *Fragaria*

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With four Figures in the Text

Abstract

The leaf initiation rate in *Fragaria vesca* (var. 'Royal Sovereign') has been compared with the elongation rate of the leaf primordia at different seasons. Certain conceptions of growth correlation within the bud are presented. Experiments on the nature of elongation and emergence of primordia are described, and the causes of emergence are discussed.

Introduction

A DETAILED study of apical growth in *Fragaria vesca* (var. 'Royal Sovereign') has been undertaken to supplement other investigations on leaf growth in this plant. Both the rate of leaf production and the mean leaf size show fluctuations with season. The former has been estimated (Arney, 1953) from the interval between the emergence of successive leaves, and this data needs to be related to the number of leaf primordia remaining unemerged within the apex to be of any fundamental significance. Fluctuations in leaf size can only be related to seasonal or other causal factors if the date of initiation of each leaf is known, and this also requires a knowledge of the number of unemerged primordia within the apex.

Experimental Details

The plants used were from the M.40 virus-free 'Royal Sovereign' clone, propagated from parents obtained from East Malling Research Station. These were grown either in the college grounds at Cardiff or at Dale Fort, Pembrokeshire, the latter site being chosen for its inaccessibility to strawberry aphis and other virus vectors. Some of the plants used in 1951–2 were potted runners of certified virus-free M.40 stock from the Waterperry Horticultural School, and some runners, from the same source but uprooted from the runner beds and sent by post, were used in 1950–1.

These plants were planted in beds which had received a dressing of bone-meal at the rate of 100 g./sq. yd. A spring dressing of 12 g. KNO_3, 6 g. KH_2PO_4, 6 g. K_2HPO_4, and 12 g. $(NH_4)_2SO_4$ per sq. yd. was applied, and the beds were then mulched with a well-rotted mixture of grass mowings and dead leaves.

[Annals of Botany, N.S. Vol. XVII, No. 67, July, 1953.]

At intervals during the years 1950–2 samples of between 10 and 12 plants were removed for dissection. Only plants with a leaf just emerging from the bud were used. During the dormant period this condition could not be fulfilled and the exceptional plants with a young emerging leaf were *not* used, the remaining plants being chosen at random.

The upper part of the stem, without mature appendages, was held in a slot between two halves of a split cork in a clamp, and the older primordia were removed under a binocular microscope. The three youngest primordia were left on the apex, which was fixed, embedded in paraffin wax, and sectioned with a microtome. All sections were mounted, so that the length of the sectioned primordia could be estimated by counting the number of sections (each 15μ thick) in which each primordium appeared. Sections were stained in Haidenhain's haematoxylin using ferric chloride as mordant, and destained in picric acid; the first beginnings of the axillary meristems could then be detected as dark staining areas, while the young primordia and apical meristems were also distinguishable.

When verifying and amplifying the conclusions reached during the first season's survey the apices were not sectioned, but the primordia were dissected away and the apex, usually with only one primordium left, was excised and mounted in lactic acid and examined microscopically to decide whether one or two primordia had been left on the apex.

The length of each primordium was measured immediately after removal from the apex.

The Number of Unemerged Leaf Primordia

The survey of the number of primordia enclosed within the apical bud at different times of the year was planned as a factorial experiment, and samples from three different sets of plants were dissected on each occasion (Table I). Dissections were performed at more frequent intervals than the table indicates, but for the sake of clarity the results from adjacent intervals have been pooled where there was little difference between them.

Analysis of variance shows no significant difference between the three sets of plants in Table I, and no significant interaction between sets of plants and season. But the seasonal variation is highly significant (0·1 per cent. level). This analysis applies only to the results obtained between September 1950 and September 1951; the results for November 1951 have been added to Table I for comparison, but they were not based on figures from all three sets of plants.

The statistically significant changes in number of primordia occur during the winter and spring. The magnitude of the increase in number of primordia between the beginning of November and the end of January is almost 1·0, which suggests that most plants produce one fresh primordium during this period. Exceptionally a leaf may emerge on one or two plants during the winter period, and this probably accounts for the variation in number of primordia within the bud in late January.

TABLE I

The Number of Leaf Primordia at Different Times of the Year

All figures except those in parentheses refer to buds dissected when a primordium had just emerged from the bud.

A. Runner plants bedded out in August 1950, when 6 weeks old.
B. Runner plants transported and transplanted in October 1950, when 3 months old.
C. Old plants, bedded out as young runners in August 1949.

Date of dissection.	*A*.	*B*.	*C*.	Means of *A*, *B*, and *C*.
Sept.–Nov. 1950 . .	4·8	5·3	4·8	5·0
Late Jan. 1951 . .	(5·9)	(5·2)	(6·0)	5·7
Feb.–Mar. 1951 . .	5·3	5·0	5·5	5·3
April–May 1951 . .	4·2	3·6	4·3	4·0
July–Aug. 1951 . .	5·0	5·0	5·3	5·1
Sept. 1951 . . .	4·6	4·9	5·0	4·8
Means . .	5·0	4·9	5·1	5·0
Nov. 1951 . . .	5·5*	4·9*	5·4	5·2

Least significant difference for means of *A*, *B*, and *C*: 1 per cent. level = 0·67.

* These values are for two sets of runner plants, bedded out at 6 weeks old in August 1951. They are not included in classes *A* and *B*.

When active leaf emergence commences again at the end of February, the rate of emergence appears to outstrip the rate of initiation of primordia, so that there are only four primordia within the bud at the beginning of May, some plants having only three. Thereafter the rate of initiation outpaces leaf emergence so that nearly all buds contain five primordia by the beginning of July. These trends have been confirmed by observations on young runner plants during a subsequent season. The mean number of primordia per bud were:

Nov. 9, 1951	5·1	15	apices dissected.
Jan. 29, 1952	6·0	12	,, ,,
May 5, 1952	4·0	15	,, ,,
May 26, 1952 . . .	4·2	15	,, ,,
June 16, 1952 . . .	5·0	6	,, ,,

Since the observed variations in number of unemerged primordia within the bud might be brought about by alterations in bud size, the seasonal changes in length of the emergent primordium have been considered, the data being given in Table II. Analysis of variance shows that there is no significant difference in the length of the second oldest primordium, but that there is a significant change in the length of the emergent primordium during the season. In August and September there is a fully significant (1·0 per cent.) increase over the spring and summer level. The values for January and February in Table II are not comparable with the other values as they are for unemerged primordia in buds which are still dormant (as far as leaf emergence is concerned). Similar dormant buds dissected on January 29, 1952, showed a length of 19 mm. for the unemerged primordia, while emerging primordia on other plants at the same time gave a value very similar to the March–July

TABLE II

Seasonal Changes in Length of Leaf Primordia (1951 Season)

Mean values of between 5 and 12 dissections; lengths in mm. Parentheses denote dormant apices from which no leaves had emerged for several weeks; the 'emerging' primordium in these apices was still enclosed within the bud.

A. Young runners bedded out in late July; dissected when 6–15 months old.
B. Waterperry runners bedded out in October (roots considerably disturbed); dissected when 5–14 months old.
C. Old plants dissected when 18–27 months old, in the same year as *A* and *B*.

		Late Jan.	Late Feb.	Mar.	May.	July.	Aug.	Sept.	Mean for season.
'Emerging' primordium	*A*.	(22)	(20)	27	28	26	32	31	27
	B.	(19)	(16)	27	24	28	34	42	27
	C.	(21)	(22)	33	28	34	34	33	29
Mean of *A*, *B*, *C* (Least significant difference = 6·0)		(21)	(19)	29	27	29	33	35	28
Second primordium	*A*.	(13)	(11)	14	9	9	12	11	11
	B.	(9)	(9)	12	6	11	12	13	10
	C.	(12)	(10)	17	9	13	12	9	12
Mean of *A*, *B*, *C* (*No* significant differences)		(11)	(10)	14	8	11	12	11	11

value in Table II, showing that the length of the emergent primordium remains practically constant during winter, spring, and early summer.

The difference in the mean length of the emerging and next oldest primordium in November 1951 and January 1952 is:

Emerging primordia:	Nov. 6, 1951	36 mm. (Least significant difference
	Jan. 29, 1952	26 mm. = 4·8.)
Second oldest primordium:	Nov. 6, 1951	15·5 mm. (Least significant difference
	Jan. 29, 1952	16·7 mm. = 3·0.)

This indicates that the increased length of the emergent primordium may be maintained through the late autumn until the last leaf has emerged. The stipules of this leaf are shorter than those of previous leaves, so that the spring leaves emerge at an earlier stage of elongation. Variations in size of the emergent primordium at different seasons of the year are clearly a function of the size of the stipules of the next oldest leaf, and of the length of the internode between these two leaves. There is no correlation between the size of either the emergent primordium or the second oldest leaf primordium and the time interval since the previous leaf emerged, or the mean rate of leaf production of the crown prior to dissection. Hence the greater length of the stipules and emergent primordia is not associated with greater vigour of the apex.

The variations in size of the emergent primordium which are indicated above are not sufficient to account for variations in the number of primordia

within the bud during the spring. If the normal size gradation is to be maintained, the emergent primordium would have to be more than twice the normal length at emergence in order to allow one extra leaf primordium to be accommodated within the bud. Variations which occur between individual crowns at any one season of the year also point to this conclusion. All those buds which contained an abnormal number of primordia have been classified into two groups—those with more than the normal number of primordia for the season, and those with less than the normal number. There were 9 of the former and 6 of the latter; the approximate equality in numbers indicates that the emergence of the oldest primordium is not immediately associated with the initiation of a sixth primordium at the apex. The individual primordium ratios (both the 'initial' and the mean ratios for the oldest four primordia) and the length of the emergent and the youngest primordium for all members of each group were meaned, giving the following results:

	Primordium length		Ratio of primordium lengths	
	Emergent.	Youngest.	'Initial'.	Mean of first four primordia.
Buds with fewer primordia than normal	32 mm.	0·28 mm.	3·9	3·0
Buds with more primordia than usual	31·3 mm.	0·08 mm.	5·7	2·2

These results confirm that the size of the bud has nothing to do with the number of primordia enclosed in it. From this conclusion it follows that the difference in size of successive primordia must be less than the average in buds with more than the normal number of primordia, and more than the average in those buds with less than the normal number of primordia; this is shown in the figures above.

The youngest primordium in those buds with more than the normal number of primordia is much smaller than in those buds with less than the normal number, and is usually hardly developed beyond the stage of initiation. Thus the sixth primordium has only just developed in these abnormal buds, and if the buds had been dissected a few hours earlier they would have contained only the normal number of primordia—5. This situation, in addition to the smaller size difference between successive primordia, accounts for the extra primordium contained in these buds. The figures show that the same two factors in reverse account for the smaller number of primordia in the buds of the other class. Thus these two exceptional types of bud represent those individual crowns in which the relationship between leaf initiation rate and relative growth rate of the primordia is stabilized (possibly only temporarily) at an initiation/growth proportion removed from the normal.

Elongation Growth of Primordia

The change in length of leaf primordia during development, from the end of the first plastochrone 10 days after initiation to the end of the phase of

rapid elongation which accompanies the unfolding and expansion of the leaf after emergence, is shown in Fig. 1. The values for primordium lengths up to 60 days old were obtained by measuring the lengths of successive primordia removed from buds in August, and are based on the means of 24 buds. The

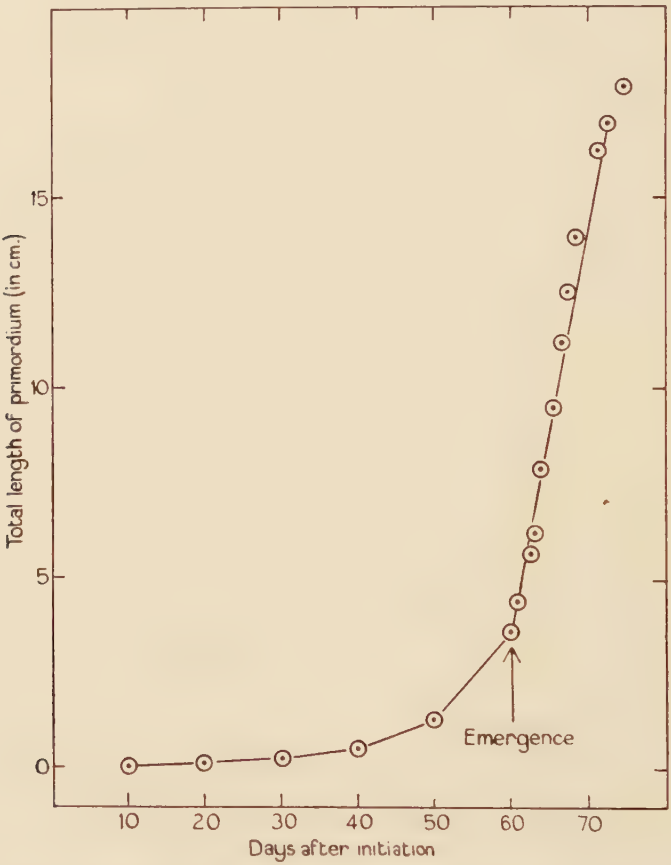

FIG. 1. Elongation of leaf primordia from initiation to maturity (during August and early September).

leaf lengths between 60 and 80 days were obtained by daily measurement of 8 young leaves which emerged on August 17. There is a complete contrast between the two phases of elongation, for the elongation rate of primordia within the bud increases exponentially (see Fig. 3), while the elongation rate after emergence remains constant, and is much higher than would have been achieved if the rate had continued to increase exponentially. Elongation after emergence is plotted on a more extended time-scale in Fig. 2, with data from another set of leaves which emerged at the end of August; this shows that the values fit closely to a straight-line relationship until elongation begins to slow down as maturity is reached.

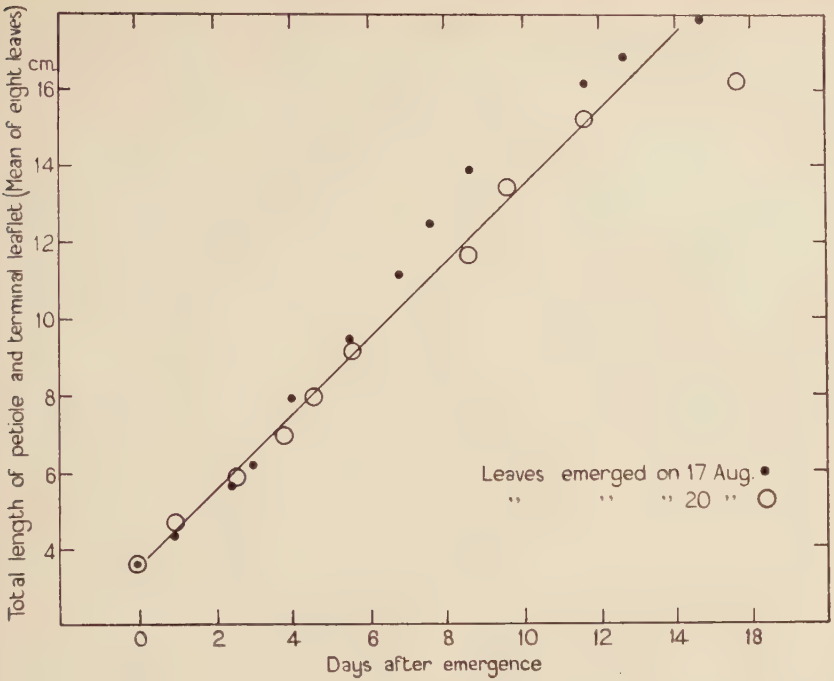

FIG. 2. Elongation of leaf primordia after emergence.

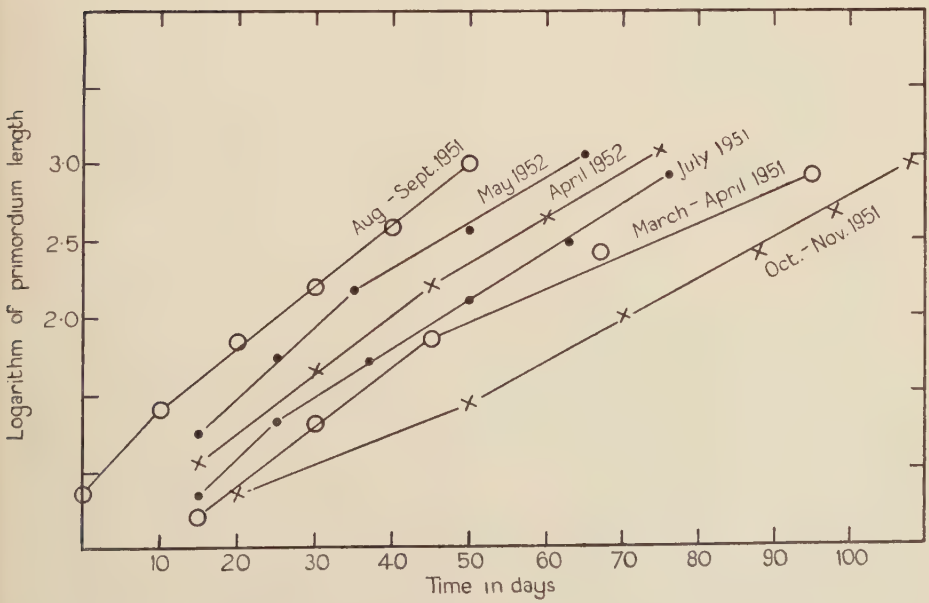

FIG. 3. Elongation rates of leaf primordia at different times of the year.

In order to demonstrate the existence of an exponential phase of growth in primordia enclosed within the bud, the logarithms of primordium lengths are plotted against age of the primordia in Fig. 3. For each of the seasons represented the points are the means obtained from at least 10 buds. The straight lines are not necessarily the lines of closest fit, but have been drawn to show as clearly as possible the closeness with which certain points do fit a straight-line relationship. It will be shown in a following section that the relative growth-rate of the very young primordia falls considerably during the first two plastochrones after initiation, at all seasons of the year; this tendency is shown in Fig. 3 in the results for July, August, and September, in which the primordia have all developed during a period of relatively constant environmental conditions. The other four sets of points represent buds in which the successive primordia have developed in changing climatic conditions. The younger primordia in the buds dissected in March, April, and May have been developing in warmer conditions than the average for the whole of the developmental period of the older primordia in the same buds; it would be expected, therefore, that the slope of the line would not be so great at the older end. The opposite is the case for buds dissected in November, and here the slope of the line is greater for the older primordia.

Although this was not the primary object, the data do give some indication of the effect of climatic conditions on the growth rate of primordia. The relative elongation rate (defined as $100\Delta L/L.\Delta T$ by analogy with the relative growth rate of West, Briggs, and Kidd, 1920) is indicated by the slope of the lines in Fig. 3. The lower relative elongation rates tend to occur in the colder months of spring and autumn, and the general slope for March and April 1951 is less than for April 1952 when the air temperature was 4° higher than in March and April 1951. But the slope for the July results (mean air temp. 62·6° F.) is lower than for August (mean air temp. 59·70° F.), so that there is not a consistently close correlation between temperature and elongation rate. It may be mentioned that the rate of growth of the apical meristem (estimated by the rate of leaf production) is mainly controlled by air temperature (Arney, 1953).

The Cause of Leaf Emergence

The immediate cause of leaf emergence is the growth of the primordium to such a length that it can no longer be contained within the sheathing stipules of the next older leaf. Emergence happens to coincide with a marked increase in the elongation rate of the primordium, and it is therefore important to decide whether this increase is the cause of emergence or the result of it. This rapid elongation after emergence might well be a process of vacuolation without cell division, since the rate of elongation remains constant over most of the period, and does not increase with increased length of the leaf. Measurements of the length of cells in the emerging and in the mature petiole showed that while the petiole length increased by 5·6 times between emergence and full expansion, the lengths of the cortical cells increased by 5·1 times between

these two stages. Thus the elongation which occurs after emergence is almost entirely a process of cell vacuolation.

It seemed possible that this change from cell division to cell vacuolation at the time of emergence might be caused by exposure of the primordium to light, or increased aeration, as a result of emergence. In order to test this possibility plants which had produced a leaf several days previously were chosen, with the expectation that another leaf would emerge in 4–5 days' time. These unemerged primordia were exposed by cutting away the stipules of the last emerged leaf, and daily length-measurements were made. The results are summarized in Table III.

TABLE III

Daily Increments in Length of Leaf Primordia laid bare approximately 4 Days before Emergence is due

	Day	1	2	3	4	5	6	7
Change in length (in mm.)	Exposed	0·7	1·5	1·0	1·5	3·5	3·0	3·0
	Control	—	—	—	—	3·5	4·0	3·5

The uncovering of the primordia did not immediately produce a rate of elongation comparable with that to be expected from normal emerged primordia, but there is a sudden increase in elongation rate at the time when the primordia would have been expected to emerge had they not been exposed; the increased rate is, moreover, comparable with that of control primordia after emergence. In other experiments slightly older primordia were laid bare, and the increase in elongation then occurred sooner after exposure, at the time when the primordia would normally have emerged. These results show that the physical consequences of emergence—increased light intensity, aeration, evaporation—are not the causes of the great increase in elongation rate after emergence.

The angular divergence between successive leaves on the crown is approximately 137°; this means that every sixth leaf is situated almost directly above an older leaf on the same stem, while none of the intervening leaves is situated anything like so closely in the same vertical line. Since a strawberry crown rarely has more than a dozen living leaves at any one time, no leaf is exactly below another leaf on the shoot, but the sixth leaf comes very near to this position. Hence it is generally true to say that when a fresh primordium is initiated at the apex, the leaf nearly vertically below it emerges from the bud and commences rapid vacuolation; for the buds typically contain 5 primordia plus an emerging primordium. But this sequence is not maintained in the February and May buds, which have 6 and 4 primordia respectively. Some experiments have been performed to determine whether the initiation of a fresh primordium did stimulate the elongation and emergence of the primordium almost directly beneath it. Buds in which a primordium was due to emerge within about 2 days' time were dissected to remove the apex and

younger primordia, but leaving the oldest primordium intact. This primordium always elongated and expanded to a considerable extent, but the situation appears to be complicated and further experiments are planned. It is nevertheless clear that the emergence of each primordium from the bud is not caused primarily by the initiation of a fresh primordium almost directly above it on the apex. Thus neither the exposure of the primordium after emergence nor the initiation of a fresh primordium above it on the apex are the causes of the change from cell division to vacuolation which brings about the rapid elongation of the emerged primordium. Possibly this change is an autonomic response of the primordium at a certain stage in its development. There is no basis yet for deciding whether the increased rate of elongation occurs just before or just after emergence, or whether there is any regularity in the sequence of events; but almost certainly there is no causal relationship between emergence and this accelerated elongation of the primordium.

The Primordium Length Ratio

The analysis of the data on elongation of primordia can be carried a stage farther by calculating the ratio of the length of each primordium to the length of the next younger primordium on the same apex; these ratios will be called the 'primordium length ratios'. The significance of the primordium length ratio can be demonstrated from the concept of oscillation in bulk of the 'bare apex' of the stem tip—a concept which has been developed in an analysis of phyllotaxis by Richards (1951), who uses the term 'bare apex' to distinguish that central and terminal part of the apex on which primordia have not yet been initiated. If V_o is the minimal volume to which the 'bare apex' is reduced after the initiation of a leaf primordium, and V_i is the maximal volume to which this 'bare apex' grows before the initiation of the next primordium, then the plastochrone interval can be defined as the time taken by a mass of cells of volume V_o to grow to a volume V_i by cell division. The length of the plastochrone will depend upon the effect of the environment on the rate of cell division, and will be of such a duration that a given mass of meristematic cells will always increase to V_i/V_o of their previous volume, provided that they have the same capacity for cell division as the cells of the apical meristem. If, under these conditions, the rate of cell division in the leaf primordium is the same as in the 'bare apex', the primordium volume ratio will be equal to V_i/V_o, and the primordium length ratio might be expected to approximate to $\sqrt[3]{(V_i/V_o)}$. If the rate of cell division in the leaf primordium is less than in the 'bare apex' under the same conditions, then the primordium volume ratio will be less than V_i/V_o, but will remain constant over successive plastochrones, in spite of variations in environment, since the length of the plastochrone is adjusted automatically to cancel out effects of varying environment on cell division rate.

Hence the primordium length ratio can be expected to remain constant, and independent of the environment, provided (1) that the elongation of the primordium is brought about by cell division, (2) that the rate of cell division

does not show a progressive change during the development of the primordium, and (3) that the rate of cell division is controlled by environment in exactly the same way in both apical meristem and primordia. The assumption that the ratio V_i/V_o remains constant under various environmental conditions seems to be justified on the theory of phyllotaxis put forward by Richards, since the ratio could hardly alter without a corresponding alteration in the ratio of the bulk of the 'bare apex' and the nascent primordium, which would be reflected in an alteration in the phyllotaxis of the shoot.

Table IV

Primordium Length Ratios

The ratio between the lengths of successive primordia at different seasons. The first primordium is the primordium just initiated and the 'initial' ratio is the ratio between the second and the youngest primordium. The 'floral' ratio is the ratio between the leaf primordia immediately before and after the inflorescence.

	Primordium ratios							Rate of leaf formation	
Date.	6th 5th.	5th 4th.	4th 3rd.	Mean of intervals 3 to 6.	3rd 2nd.	Initial ratio.	Floral ratio.	Difference in age of primordia.	Emergence interval.
1951									
Jan. . .	1·8	2·1	2·2	2·0	3·0	3·7	4·7	>30 days	>30 days
Mar. . .	2·1	1·9	2·6	2·1	2·6	3·6	4·1	30 days	22 days
May . .	3·4	4·2	3·6	3·7	..	5·4	—	14 days	14 days
July . .	2·8	2·5	2·3	2·55	2·8	3·9	—	9 days	9 days
Aug.–Sept. .	2·8	2·4	2·5	2·6	2·6	4·1	—	9 days	9 days
Nov. . .	2·3	2·0	2·4	2·3	2·7	4·1	4·5	18 days	18 days
1952									
Jan. . .	1·5	1·8	1·9	1·7	2·8	—	3·0	>30 days	>30 days
May . .	3·1	2·8	3·1	3·0	—	4·0	—	11 days	11 days

Table IV gives the values of the primordium length ratios at various times of the year; each value is the mean of the comparable primordium length ratios from each of the individual buds dissected on any one occasion. Analysis of variance shows that the 'initial' and the 'floral' ratios are significantly (1 per cent.) higher than the others, and that the May values are higher than for the other months. With these exceptions, all the other values, for all seasons of the year except May, show no significant differences. Thus the primordium length ratio is substantially independent of environment, and the results in Table IV therefore provide evidence in favour of the assumptions in the preceding paragraph; for it is unlikely that a departure from any one of these assumptions would be exactly counterbalanced in its effect by simultaneous departures from the others.

The 'initial' ratio is significantly greater than the others at every season of the year, and this confirms that the relative elongation rate of the primordia falls appreciably during the two plastochrones after initiation. This initial drop in relative elongation rate appears to be a gradual process; when the logarithm of the 'initial' ratio for each bud is plotted against the length of the youngest primordium the scatter diagram indicates a linear relationship, and

the correlation coefficient of −0·43 indicates a highly significant (0·1 per cent.) negative correlation between log. 'initial' ratio and the length of the youngest primordium. Thus the relative elongation rate of these primordia during the first two plastochrones after initiation decreases with increasing size (= age) of the primordium. But the decline in the relative elongation rate of the primordia does not continue after the first two plastochrones. When the log. primordium ratio for the third plastochrone (based on the lengths of the second and third youngest primordia) is plotted against primordium length, the slope is barely perceptible, and there is hardly any change in primordium length ratio with increasing length of the primordium.

It must be remembered that the primordium length ratios automatically compensate for changes in the environmental conditions. Hence the relative constancy of the primordium length ratios for the older primordia indicates that the elongation rate of each primordium would have been proportional to the primordium length if the measurements had been taken on primordia of different ages over the same short period of time, or over longer periods of time under constant environmental conditions. A similar proportionality between elongation rates of successive primordia and primordium length would also hold for each season of the year, since the primordium ratios remain constant throughout most of the year. The elongation rate would therefore increase exponentially with age of the primordia at every season of the year, if environmental conditions remained constant throughout the period of development. The lack of such an exponential relationship in the spring and autumn (the points in Fig. 3 do not fall on a straight line) is therefore the result of changes in the environment during the development of the older primordia measured.

The primordium length ratio for the primordia developed immediately before, and immediately after, the inflorescence is given in column 8 of Table IV as the 'floral ratio'. The transformation of the apex into an inflorescence would be expected to delay the initiation of the next leaf primordium which arises in the axil of the last leaf initiated before the inflorescence, and so the larger primordium length ratio is to be expected in this case. Although there is this gap in the size sequence of the leaf primordia, the number of primordia in the bud is not affected because, in many cases (28 out of 48 buds dissected), there were two leaf primordia formed almost simultaneously in the axil of the leaf initiated just before the first inflorescence. The second inflorescence was followed by two equally sized leaves in almost every case. This rapid formation of two successive leaf primordia makes up for the longer interval between the initiation of the primordium next before, and the primordium next after the inflorescence, and so, in spite of the sympodial growth of the crown in floral initiation, the overall rate of leaf production during this period is the same as in the preceding vegetative period. The two leaf primordia of approximately the same age remain the same size throughout their development, but the difference between the size of these two leaves and the size of the leaf immediately preceding the inflorescence remains much

greater than that between successive primordia in the vegetative bud until after emergence. Thus the production and growth of the inflorescence does not seem to affect the relative elongation rates of the adjacent leaf primordia.

The marked increase in primordium length ratios in May has been observed for two successive seasons, and is clearly associated with the smaller number

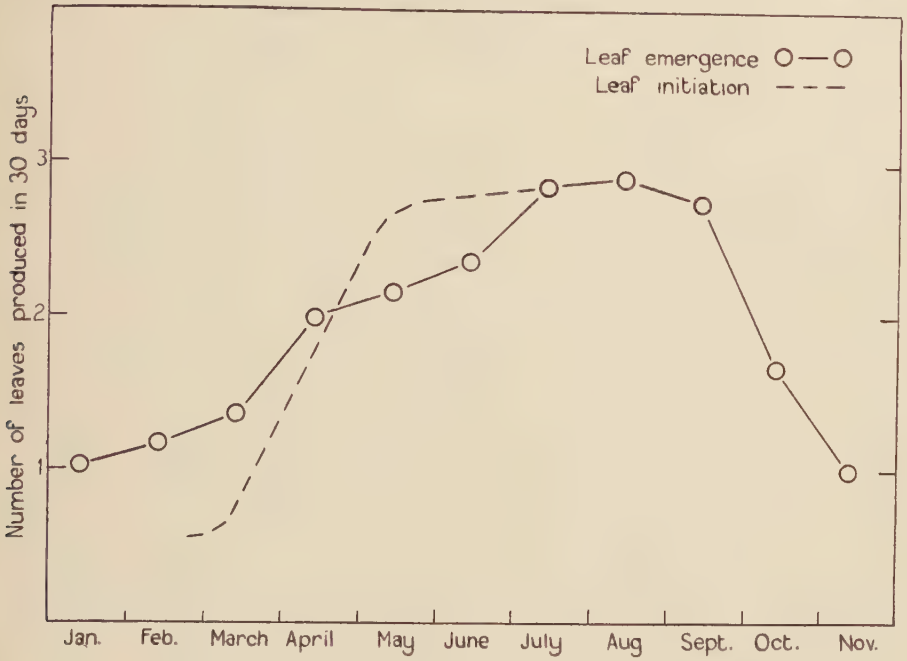

FIG. 4. Rates of leaf initiation and leaf emergence in 1951.

of primordia within the bud at this time of the year. Since the emergent primordium is no shorter in May, the primordium length ratios are bound to be higher if there are fewer primordia within the bud. The situation is the result of an increase in the elongation rate of the primordia relative to the initiation rate; the relative elongation rates for May are nearly as high as the rates for the summer months (Fig. 3). Hence the peculiarity of the May buds is the result of the combination of a low rate of initiation (Fig. 4) with a high rate of elongation of the primordia. Since the primordium length ratios and the number of primordia within the bud become normal by the end of June, the rate of leaf initiation must have caught up with the high rate of elongation by this time (Fig. 4).

DISCUSSION

The characteristic number of primordia enclosed within the buds of these strawberry plants is obviously five (see Table I); this number is maintained from the beginning of June until November or December, and it is only during late winter and early spring that there is any general departure from

this condition. Thus the rates of leaf production based on the time of leaf emergence are the same as the rates of leaf initiation during the whole of the summer and autumn, but are higher in March and April, and lower in June, than the rates of leaf initiation for the month. The approximate monthly mean rate of leaf initiation has been estimated for the 1951 season, and is compared with the monthly mean rate of leaf emergence for the same season in Fig. 4. But data must be obtained at more frequent intervals throughout the winter and spring before the rates of leaf initiation and emergence can be compared in detail.

The relative elongation rate of the primordia and the leaf initiation rate (which accurately reflects the growth rate of the apical meristem) vary widely at different seasons of the year (Fig. 4), and yet the proportion between the two, which is indicated by the primordium length ratios, remains almost the same except in May. It has already been explained that this constancy of relationship would be expected on the assumption that the growth of both primordia and apex are mainly a matter of cell division. The relative constancy of the primordium length ratio for all but the youngest primordia in the bud shows that the proportion between initiation rate and relative elongation rate of the primordia is held fairly constant even over the temporary fluctuations in rates which may occur between successive plastochrones in individual crowns. The variance of the primordial ratio within each bud taken separately is no greater than the residual variance, and if, as would be expected, the residual variance represents the experimental error in the determination of primordium lengths, the actual variations in relative elongation rate between the older primordia within the bud may be much smaller than the figures in Table IV would suggest. Records show that if the emergent primordium in any bud is longer than the average for the season, all the other primordia within that bud will tend to be longer than the average, and vice versa for buds with an emergent primordium shorter than the average. Thus the relative lengths of successive primordia in each bud are controlled more closely than the standard errors might indicate.

The data of Table IV and the correlation between the youngest primordium length and the 'initial' ratio indicate that the relative elongation rate of the youngest primordium is at least twice as great (for any given rate of initiation) as the relative elongation rate of the older primordia. But, clearly, the high relative growth rate of the youngest primordium is as closely linked to initiation rate as is the lower relative growth rate of the older primordium, since the 'initial' ratio remains fairly constant over the whole season.

Richards (1951) has calculated from Wardlaw's data on *Dryopteris aristata* that the average cell in the interior of the apical cone remains quiescent between successive cell divisions while three leaf primordia are being initiated on the surface of the apex. Thus it appears that the rate of cell division is not uniform in all parts of the apex, the leaf primordia being regions of especially active cell division. There is every probability that the rate of cell division in the very young leaf primordia may be at least as high as that of the

apical cells themselves, but as the leaf primordium ages its overall cell division rate declines as the cells become more and more vacuolated. All primordia except the two youngest are seen to be composed of vacuolating and dividing cells, in contrast to the purely meristematic cells of the young primordium, which stain far more heavily with haematoxylin, and so the rapid fall in relative elongation rate in the young primordium occurs at the same time as the cells of the primordium are changing from the purely meristematic to the 'rippon meristem' condition.

Summary

1. As each leaf primordium emerges from the sheathing stipules of the next older leaf there are five younger primordia already differentiated at the stem apex. An extra primordium is formed during the winter, while primordia rarely emerge at this time, so that there are six enclosed primordia at the beginning of February. Leaf emergence outpaces leaf initiation during early spring so that most plants have only four primordia at the beginning of May. By the end of June five enclosed primordia are present, and the number remains at five until the end of November.

2. The rate of leaf initiation has been calculated from the rate of leaf emergence and the number of unemerged primordia. The two rates are equal from July to November, but leaf initiation exceeds leaf emergence in the winter, and lags behind leaf emergence in the spring.

3. The elongation rate of the primordia increases exponentially after the first two plastochrones, until emergence, but the relative elongation rate of the freshly initiated primordium falls markedly during the first two plastochrones. The latter stage corresponds with a phase of purely meristematic cell division, and is followed by a 'rippon meristem' condition; in both stages elongation is brought about almost entirely by cell division. At emergence the elongation rate increases suddenly and is maintained at the same high level until expansion is almost complete; this elongation is entirely a process of cell vacuolation.

4. Cell division of the enclosed primordia and of the apical meristem is equally affected by seasonal changes in environmental conditions.

5. The number of primordia enclosed within the bud appears to be the result of a particular balance between initiation rate and growth rate of the primordia, and the number of primordia remains constant because initiation rate and growth rate of the primordia are so similarly affected by seasonal conditions. The actual proportion (and hence the number of primordia) may vary between individual plants, a few having six, and a few having four enclosed primordia.

6. The final rapid elongation of the primordia by vacuolation is not the result of emergence. Nor is emergence the result of the initiation of a sixth primordium at the apex, although this primordium is initiated in a position approximately above the emerging primordium since the mean divergence angle between successive leaves is approximately 137°. The change from cell

division to vacuolation seems to be autonomic, depending on the stage of development of the primordium.

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The Life-History of *Marginariella urvilliana* (Ach. Rich.) Tandy

BY

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With twelve Figures in the Text

Abstract

In *Marginariella urvilliana* gametes are produced in the third year and then annually for several years. On germination of the egg a single primary rhizoid is formed. Its early subdivision into four main branches is unusual amongst the Fucaceae. Growth of both the main axis and of the receptacles is carried out by a three-sided apical cell. The oogonia develop earlier than the antheridia. Nuclear divisions are completed within the conceptacle, often several weeks before extrusion of oospheres. The liberated oosphere is held in a tubular stalk until after fertilization and the sporeling has begun to develop.

Marginariella has affinities with the other endemic submerged Australasian fucoids, and together these form a rather isolated group, probably rightly included within the Fucaceae.

Introduction

MARGINARIELLA URVILLIANA is a submerged member of the Fucales, endemic to New Zealand and found only from Cook Strait southwards. It grows off rocky coasts, usually in somewhat sheltered situations in shallow water.

In old plants the main axis may reach a length of 5 or 6 ft. and bears a distichous series of incurved, broad leafy laterals, toothed on their outer margins and bearing receptacles and spherical air-bladders on their adaxial margins. The apical cell is situated at the base of a depression and is protected by the young leafy laterals. Branching of the axis only occurs if the apex is damaged, when proliferation may result in the production of several branches each repeating the structure of the main axis. The attaching organ of the mature plant is of a type not usual in the Fucales, and consists of series of branched haptera (Fig. 2 *h*) resembling those of the Laminariales. The young plants, however, possess an unbranched, conical attaching disc of the usual fucalean type. Similar branched attaching organs are also seen in *Seirococcus*, *Phyllospora*, and *Scytothalia*, also infra-littoral members of the Fucales.

All plants investigated were from Quarantine Island, Otago Harbour.

[Annals of Botany, N.S. Vol. XVII, No. 67, July, 1953.]

Growth and Development

The youngest free-living plants collected were heart-shaped plates, in which the characteristic lobing of the apex was already evident (Fig. 1, A–C).

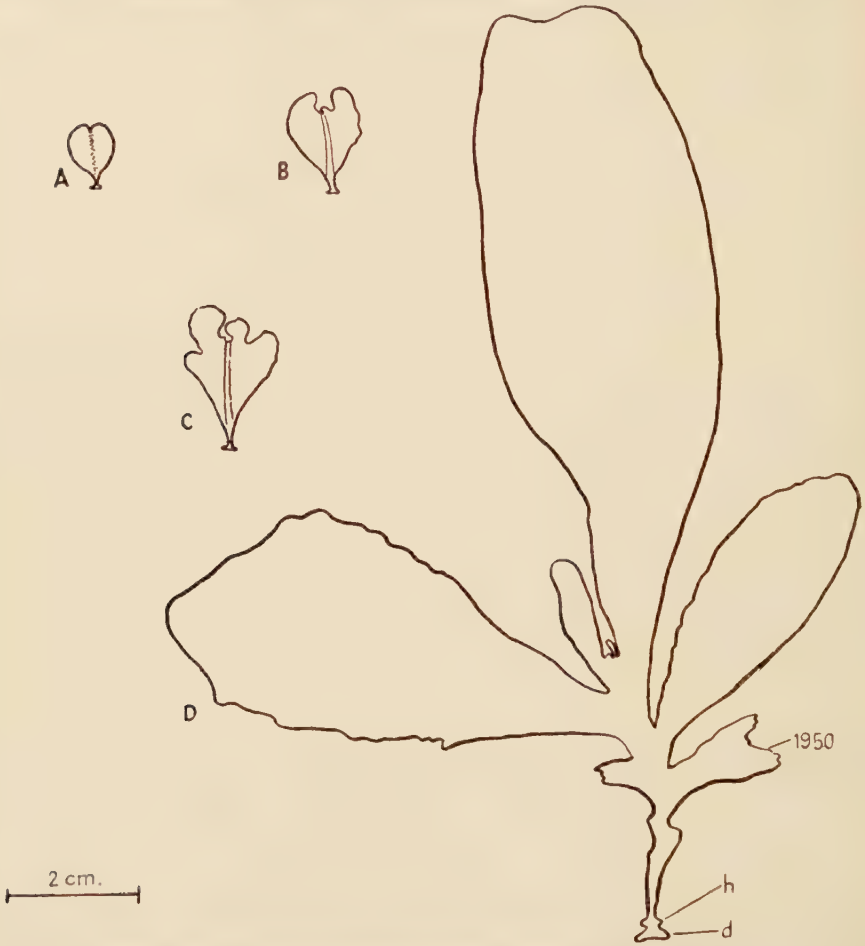

Fig. 1. Young plants of *Marginariella urvilliana* collected on Nov. 12, 1951. A–C. Plants 3 months old, showing a midrib, and the rudiments of the first juvenile leafy laterals. D. A plant 15 months old with 4 juvenile, sterile laterals. The plant is still attached by a conical attaching disc (*d*), above which the first haptera are just beginning to appear (*h*). The date indicates the year in which the lateral was laid down.

These plants, collected in November 1951, were estimated to be between 3 and 6 months old, as the period of fertility for 1951 had lasted from the middle of May until early August.

During the second year the plant produces a main axis, growing by means of an apical cell, and bearing a distichous series of leafy laterals which are purely vegetative (Fig. 1, D), and which have a characteristic juvenile appearance, being short and broad.

After the production of a number of these juvenile leafy laterals—probably at the beginning of its third year—adult leafy laterals are produced with receptacles and air-bladders along their adaxial margins (Fig. 2). Almost from

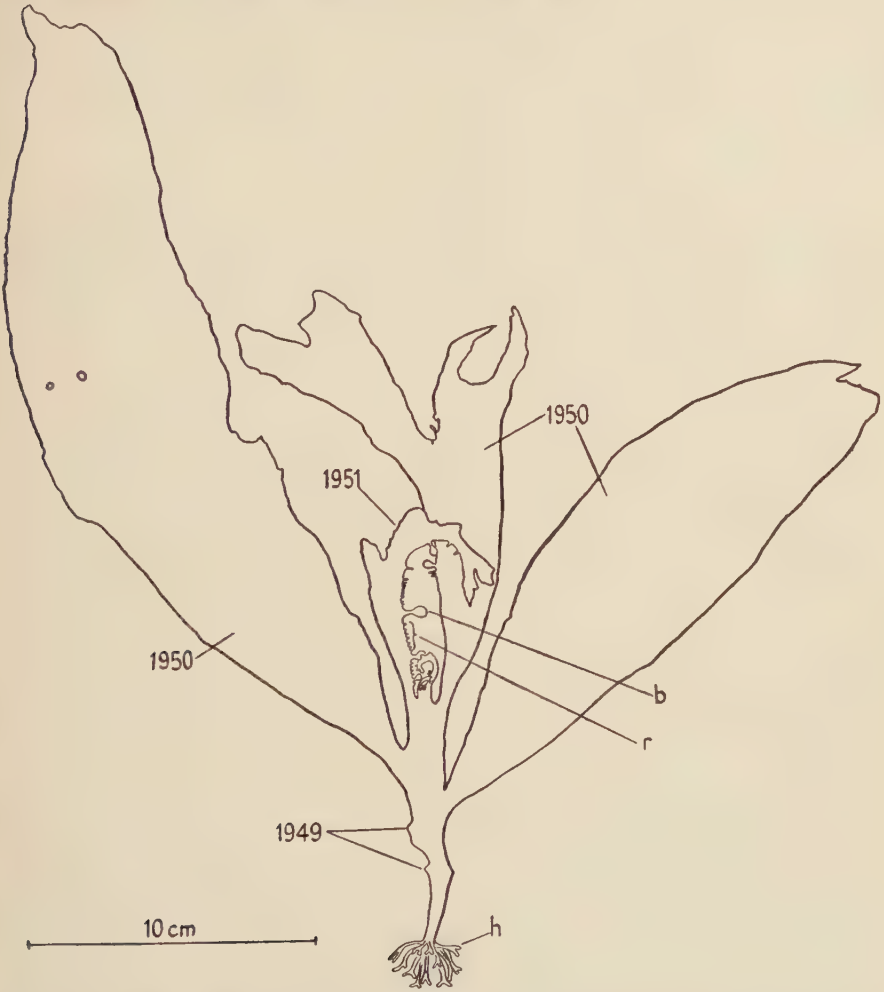

Fig. 2. An older plant collected on Nov. 12, 1951. This plant is entering its first season of fertility, and is estimated to be 2 years 3 months old. The newly forming laterals bear on their adaxial margins air-bladders (*b*) and developing receptacles (*r*). The holdfast of branched haptera (*h*) has now replaced the conical disc. The dates indicate the years in which the laterals were laid down.

their first inception these fertile laterals can be distinguished from the juvenile ones, as they are tightly inrolled in their early stages of development, whereas the juvenile laterals are not (cf. Fig. 1, D). The receptacles take about a year to mature, so the plant is 3 years old by the time it produces its first crop of gametes.

Four to six such fertile laterals are produced annually, reaching maturity between May and August, maturation of the receptacles on any one lateral being more or less simultaneous. After the gametes are shed, first the receptacles and then the laterals die back or are worn away, whilst a further crop is produced the following year on the new laterals. In the oldest plant examined scars of at least twelve laterals were visible, together with the current season's growth of four laterals and the following season's laterals just beginning to unfold, giving an estimated age of 5–6 years.

Anatomy

The apical cell. The apical cell was examined in young plants of about 1 cm. in length, in mature plants, and in receptacles. In all cases it was three-sided in transverse section (Fig. 3, A). Longitudinal sections in both planes

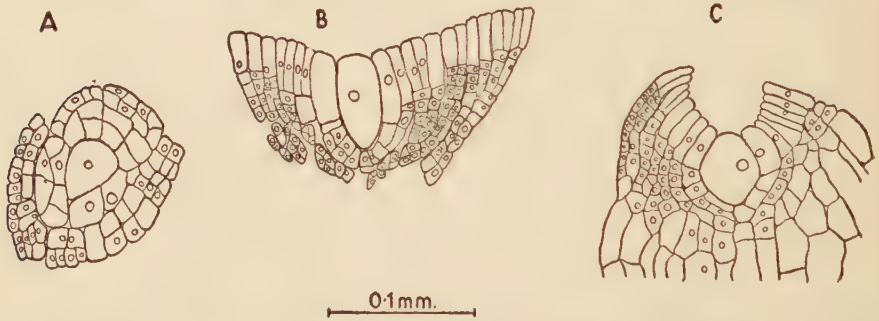

Fig. 3. Sections through the apical cell of the main axis of a mature plant. A, in the transverse plane. B, in the longitudinal plane perpendicular to the plane of flattening of the axis. C, in the longitudinal plane parallel to the plane of flattening. A and B from apices of mature plants, C from a young plant ½ in. long. All outlines drawn with a camera lucida.

(Fig. 3, B and C) indicate that the cell is of the 'Brazil nut type' described by Dawson (1940) in *Carpophyllum flexuosum*. A three-sided apical cell of similar form was also seen in material of *M. boryana*, collected by Mrs. E. Willa of Stewart Island. This feature is of interest, since *Marginariella* is classified in the Fucaceae, a family in which the three-sided apical cell of the sporeling is characteristically replaced at an early stage by a four-sided apical cell.

The medulla. As is usual in the submerged fucoids, the medulla is a compact tissue, with no gelatinization of the cell walls, so that the filaments of medullary cells lie close to each other (Fig. 4, A). In the receptacles, however, the medullary filaments may become separated by the ingrowth of mainly horizontally running hyphae, the cells of which later become distended so that a compact, pseudoparenchymatous tissue results.

Active surface growth causes elongation of the medullary cells in such a way that their nuclei, surrounded by a little cytoplasm, remain at the upper ends of the cells just below the horizontal walls. This region of the cell retains its original diameter, whilst the middle regions collapse somewhat, so that an appearance similar to that of the trumpet cells of the laminarians

results (Fig. 4, B). This may indicate that growth of the cell wall is taking place in its upper region. The condition occurs throughout the plant and

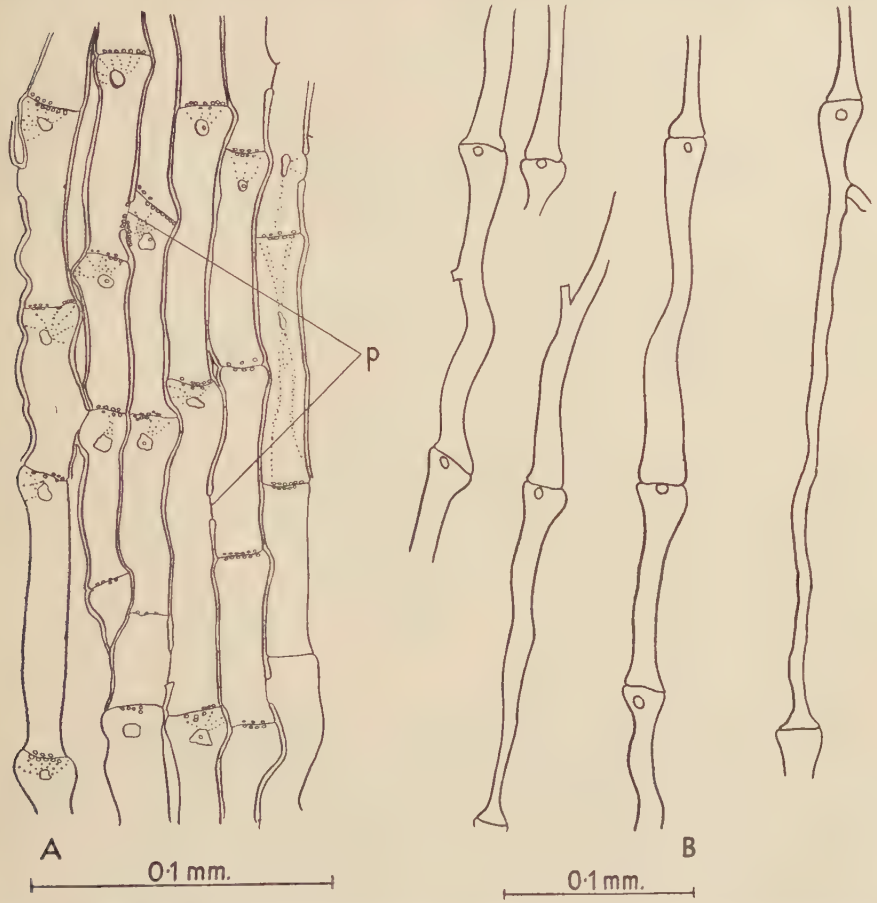

FIG. 4. Cells from the medulla showing the elongation of the cells and the position of the nuclei below the distal septum. A. Cells from the medulla of a young leafy lateral showing the compact arrangement with no gelatinization. *p* = primary pit connexion. B. Cells from a mature receptacle, to a smaller scale, showing the greater degree of elongation, and the wide separation of the filaments. The interweaving horizontal hyphae are omitted. All outlines drawn with a camera lucida.

also in *Himanthalia elongata* (Naylor, 1951), as well as in all the New Zealand species of *Carpophyllum* (unpublished results).

The Receptacles

From their first appearance in May until the time the gametes are shed the receptacles take approximately a year to develop. The receptacles develop from grooves in the adaxial margins of the leafy laterals. The groove is visible at an early stage of development, being apparent in the third rudiment

enfolding the apical pit (Fig. 5). In the still younger rudiments the forerunners of the grooves are seen as groups of meristoderm cells, rather larger and longer than their neighbours. A temporary cessation of periclinal divisions in these cells causes them to become slightly sunken beneath the

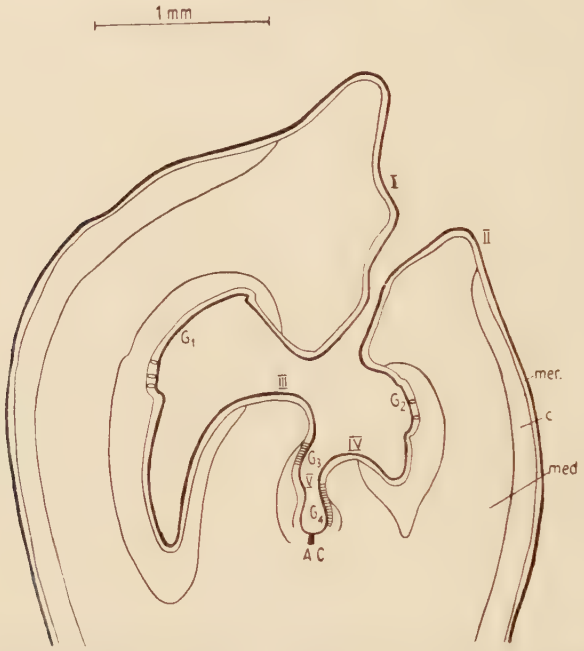

FIG. 5. A diagrammatic representation of a section in the plane parallel to the plane of flattening through an apex at the time of inception of the fertile leafy laterals. The Roman numerals I–V indicate the successive laterals cut off by the apical cell (A.C.) which is lodged at the base of a depression and protected by the inrolled rudiments of these laterals. In the laterals III and IV the regions G3 and G4 indicate regions of large cells with less dense contents than their neighbours, and which eventually form the lining to the groove (G1 and G2) from which the receptacles later develop. Some of these cells remain large and retain their meristematic capacity and become the initial cells of the receptacles. Three such cells are indicated in G1 and two in G2. Outline drawn with a camera lucida. *c* = cortex; *med.* = medulla; *mer.* = meristoderm.

surface. At intervals along the groove isolated meristoderm cells remain large and constitute the initials by whose activity the receptacles are formed. These cells are three-sided in transverse section and biconvex in longitudinal section, as is the initial of the main axis.

During the early stages of development the receptacles are cylindrical with the apical cell in a small, mucilage-filled pit; but after the conceptacle initials have been laid down, the pit and apical cell are lost and the sterile, pointed tip develops. The first formed receptacles attain their full length by about December, but the conceptacles and gametes are immature, and the adult anatomical structure has not developed.

Anatomy. As the receptacles increase in length and diameter, the filaments

of medullary cells become widely separated, and often at this stage the centre of the receptacle may contain gas-filled spaces, thus closely resembling the early stages of development of the bladders. In the air-bladders, however, these spaces become more and more distended, eventually uniting to form a large central cavity, but in the receptacles they soon become occluded by interweaving horizontal hyphae. The hypal cells swell and form a compact, pseudoparenchymatous core in a manner similar to the formation of the central blocks of pseudoparenchyma in the receptacles of *Himanthalia lorea* (Naylor, 1951). The characteristic appearance of the medullary cells themselves has already been noted (Fig. 4, B).

The Conceptacles

The conceptacles are initiated at an early stage in the development of the receptacles and are already visible in August in receptacles which are no more than 1 mm. long.

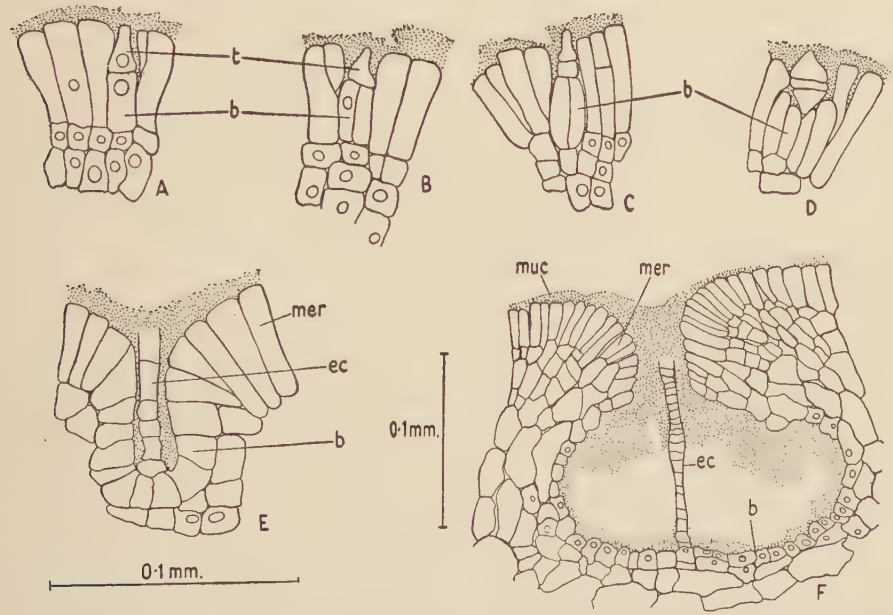

Fig. 6. Stages in conceptacle development. A–D, stages still within apical groove. E, from near tip of young receptacle. F, the most mature conceptacle from a receptacle about 5 mm. long. All outlines drawn with a camera lucida. *b* = basal cell; *ec.* = ectocarpoid hair formed from tongue cell; *mer.* = meristoderm; *muc.* = mucilage; *t* = tongue-cell.

The conceptacle initial can be seen whilst still within the apical groove, as it is somewhat larger than the neighbouring meristoderm cells and is slightly sunken beneath the surface. Shortly after it passes beyond the apical groove it divides by a transverse wall, cutting off a tapering tongue-cell from a broader basal cell (Fig. 6, A). The basal cell divides repeatedly by anticlinal walls (Fig. 6, B–F) and gives rise to a single layer of small cells with rather

deeply staining contents and prominent nuclei. These line the conceptacle, except in the region of the ostiole where the meristoderm cells form the lining. The tongue-cell divides by a series of periclinal walls to give a uniseriate filament reaching to the ostiole and which persists until the conceptacle is about 165 μ in diameter (Fig. 6, F) but which has disappeared by the time the oogonia begin to develop. At first the cavity of the conceptacle is flask-like, but as the receptacle increases in length it becomes elongated in the longitudinal direction.

The conceptacles have been hermaphrodite in all the material I have collected. The oogonia, each containing a single oosphere, are separated by clusters of moniliform hairs with short, branched antheridial hairs (Fig. 7, F) at intervals between them. Gourley (M.Sc. thesis, unpub.), however, reports plants collected in the Christchurch district to be dioecious. I have also seen preserved material which was dioecious, but it had no locality marked, and I have been unable to find such dioecious plants in the field.

The Oogonia

(*a*) *Development.* The oogonia first appear as papillae projecting from the lining layer of the conceptacles. This stage of development was normally reached in December, but occasionally as early as October. The oogonium then enlarges until it is an ovoid body about 265 μ long and 110 μ broad. At this stage it is characterized by a conspicuous central nucleus (Fig. 7, A–F). Nuclear divisions and differentiation of the oogonium wall then follow, most of the oogonia in any one receptacle being at approximately the same stage of development.

The wall becomes several-layered and the cytoplasmic contents take on a characteristic amphora shape. From the living material it was possible to see more detail of the oogonium wall than Delf (1937) was able to see in herbarium and preserved material alone. The distal collar of more highly refractive material is continuous at its inner end with the swollen layer immediately surrounding the oosphere, and its outer end is prolonged to form a fine layer between this swollen layer and the exochite (Fig. 9, B).

(*b*) *Cytological maturation.* Receptacles of *M. urvilliana* of varying ages were fixed at intervals throughout 24-hour periods on several occasions covering a period of 2 months, extending from a fortnight before any signs of extrusion were observed on the shore until well into the period of maximum fertility of the plant. The fixatives used were Abe's osmic acid fluid and that of Karpechenko. The sections were 8–10 μ thick and stained with Heidenhain's iron alum haematoxylin. However, no stages of nuclear division were seen.

During the early stages of enlargement of the oogonium there is a corresponding enlargement of the nucleus, although, as in *Cystoseira barbata* (Nienburg, 1910), there is little change in its structure. It is characterized by a large nucleolus and a uniform, granular structure. When the oogonium is about half-grown the nucleus attains its maximum size. At this stage (Fig. 8, A) numerous deeply staining chromatophores become aggregated around

the nucleus, and thread-like regions within the nucleus stain very intensely. The next stage seen after this prophase shows four small nuclei lying in a homogeneous, granular region previously occupied by the single, large central

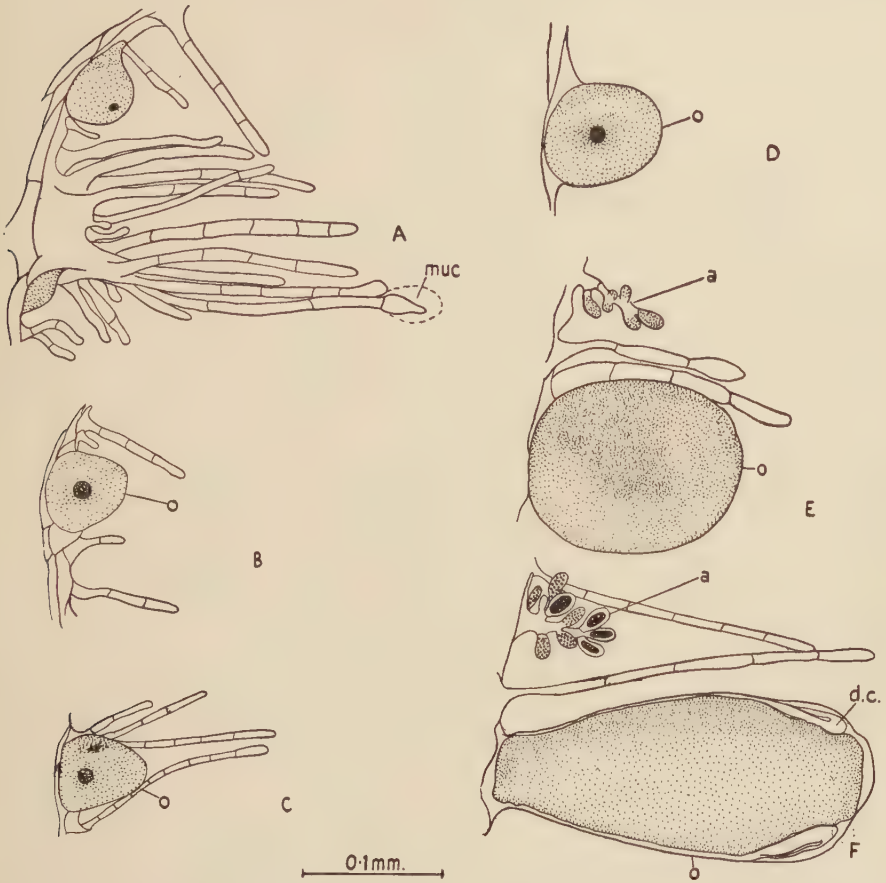

Fig. 7. Stages in development of the oogonia and antheridia taken from the most mature receptacles collected at intervals from November to March. A and B, November 13, 1950; C, December 8, 1950; D, February 5, 1951; E, April 8, 1951; F, April 22, 1951. All outlines drawn with a camera lucida. *a* = antheridial hair; *d.c.* = distal collar of mucilage in oogonial wall; *muc.* = mucilage secreted by moniliform hair; *o* = oogonium.

nucleus (Fig. 8, B), suggesting a meiotic division. Nienburg states that the spindle in the first division in *C. barbata* is intranuclear. Possibly this is the case here also.

These four nuclei travel to the peripheral region of the cytoplasm, where they enlarge (Fig. 8, C) and divide again so that there are four pairs of small nuclei (Fig. 8, D). These eight nuclei become evenly dispersed throughout the peripheral cytoplasm and enlarge once more (Fig. 8, F). One of them eventually becomes the egg nucleus and, before extrusion commences, takes

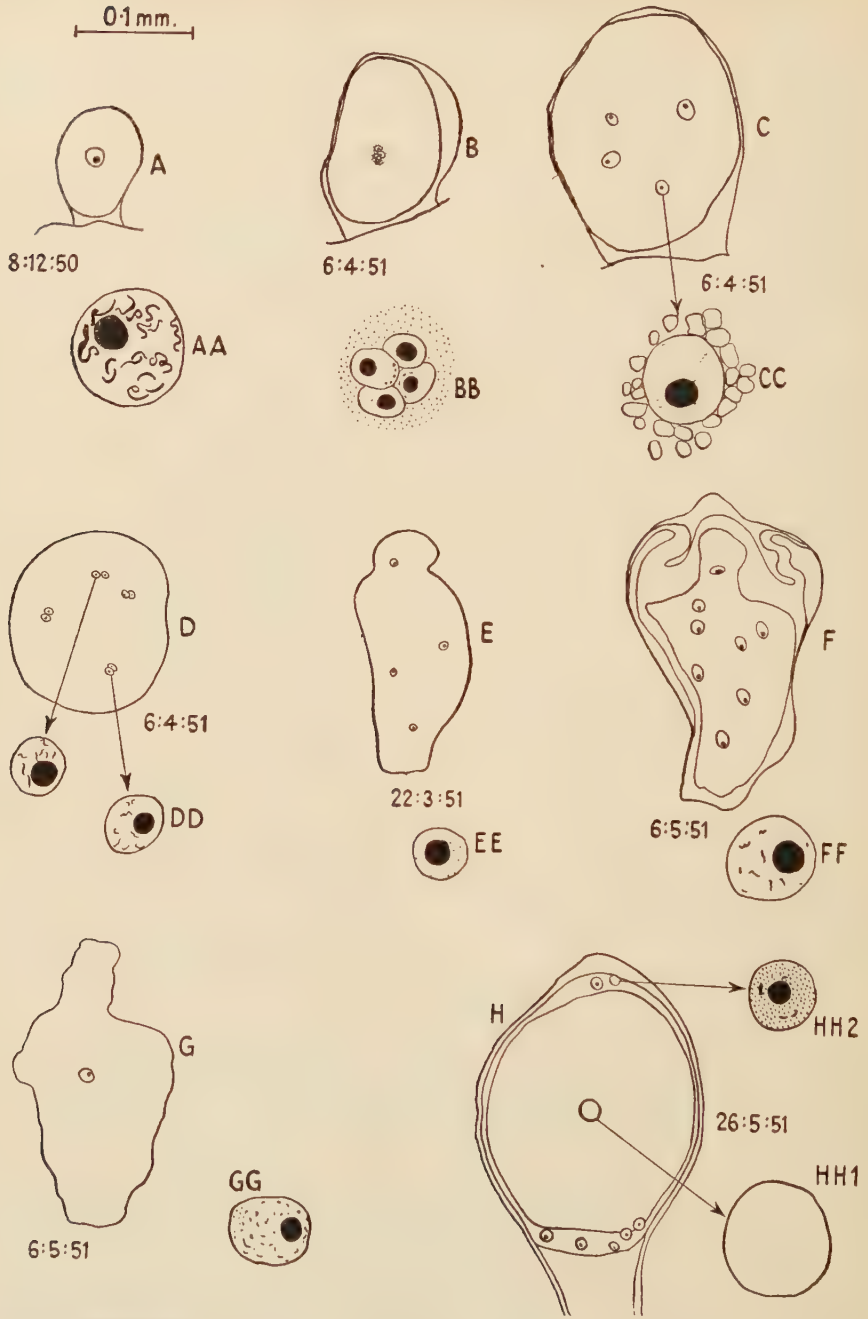

FIG. 8. Stages in the maturation of the oosphere. A–H. The entire oogonium showing the number and position of the nuclei. AA–HH, representative nuclei at a higher magnification. A–F, whilst still within the conceptacle. H, an extruded oogonium. The date of collection is indicated in each case. All outlines drawn with a camera lucida.

up a central position (Fig. 8, G). The seven supernumerary nuclei are extruded from the cytoplasm either *shortly before* the escape of the oosphere (Fig. 8, G) or *during* the process (Fig. 9, C, E, and F). Immediately after liberation, seven nuclei, still well organized, can be clearly seen between the oosphere and the mucilaginous layer of wall (Fig. 8, H).

In some cases (Fig. 8, D) the nuclear divisions are completed before wall differentiation begins, but in others (Fig. 8, E) they are not.

The first signs of nuclear division were seen in the material collected on March 8, 1951, when a few oogonia contained divided nuclei. A fortnight later many were four-nucleate, and for the next few weeks—until April 21—the majority were in this condition. Eight-nucleate oogonia were found in early April, several weeks before any extrusion was seen.

This occurrence of the first (meiotic?) division as much as 2 months before liberation, and completion of nuclear divisions in some cases several weeks before extrusion, is in contrast with the condition in *Sargassum tortile*, where the nuclear divisions begin during the afternoon preceding extrusion and are completed by the following morning when extrusion takes place (Abe, 1938).

In the liberation of a well-organized oosphere, *M. urvilliana* resembles *Coccophora langsdorfii* (Tahara, 1929), *Turbinaria turbinata* (Blomquist, 1945), *Cystophyllum sisymbrioides* (Shimotomai, 1928), and *Bifurcaria tuberculata* (Rees, 1933), amongst the stalk-forming fucoids. In many species stalk formation appears to be correlated with late maturation of the oosphere, as in *Sargassum horneri* (Kunieda, 1929), *S. tortile* (Abe, 1938), *Turbinaria fusiformis* (Tahara, 1929), *Scytothalia dorycarpa* (Naylor, 1949), *Carpophyllum maschalocarpum* (Delf, 1939), and *C. flexuosum* (Dawson, 1940), where the contents are liberated whilst still multinucleate. Occasionally degeneration of the supernumerary nuclei does not occur until after fertilization, as in *Sargassum horneri* and *Sargassum tortile*; in the latter species sometimes two nuclei are fertilized and commence division. In *Sargassum linifolium* (Nienburg, 1910) oosphere maturation is not initiated until an even later stage, the oogonial contents being released with the nucleus still in the prophase of the first division. There thus appears to be no consistency of behaviour even within a single genus. In *M. urvilliana*, however, liberation was always of a completely organized oosphere.

The Antheridia

The antheridia do not begin to develop as early in the season as the oogonia, but reach maturity at the same time. Developing antheridial hairs were first seen in March (Fig. 7, E), and these quickly developed into short, branched tufts bearing the ovoid antheridia.

Extrusion of the Oogonia

Observation of the extrusion of the living oospheres confirms the account already given by Delf from preserved material, but a study of the *living* egg

emphasizes the importance of the plasticity of the living cytoplasm during liberation.

Extrusion was watched both in detached laterals which had been kept permanently submerged and also in entire plants in deep tanks. In both cases the oogonia were discharged along the whole length of the receptacle until all were extruded, thus differing somewhat from Tahara's (1913) record for *Sargassum*. Extrusion commenced in both types of material 3 hours after its collection and continued for about 24 hours, the time of commencement coinciding with the time of high tide. Thus, possibly extrusion may be related to tidal rhythm. In collections made at low tide no plants were observed *in situ* with extruded oogonia. There appears, however, to be no correlation between oosphere discharge and the period of the spring tides, as these observations were repeated both on the spring and the neap tides, liberation occurring on both occasions.

Total submergence appears to be a necessary condition for extrusion. Material kept moist in dishes showed no extrusion, but on immersion in sea-water after varying periods of exposure up to 24 hours, discharge commenced almost immediately and fertilization occurred in a normal manner. Such a period of exposure is not, however, necessary to induce extrusion, as the plants normally occur in the infra-littoral belt, and plants carried back to the laboratory without any exposure at all effectively liberated their oospheres.

The first stage in extrusion, as seen in sections of living material mounted in sea-water, consists of the bursting of the exochite at the tip. The oosphere, surrounded by the very swollen middle layers of the wall, then passes into the cavity of the conceptacle whilst still attached to the empty exochite by the fine outer layer of the distal collar. This collar is apparently very extensible, and normally the oosphere within its mucilaginous wall, guided by the mucilage-secreting paraphyses, passes quickly out of the ostiole, becoming much constricted as it passes through the narrow ostiole. Great extensibility is also demonstrated by the way it extends in potash (Fig. 9, B).

Once in the sea-water, the oosphere, still surrounded by the mucilaginous layer, flows out of the cup formed by the top of the tubular stalk, becoming much constricted as it forces its way between the infolded margin (Fig. 9, C–F). As it escapes the seven supernumerary nuclei are sometimes actively extruded from the cytoplasm and lie in the region between the oosphere and swollen wall (Fig. 9, E). Once free, the oosphere assumes a spherical shape (Fig. 9, G), and its large nucleus remains in its central position. Occasionally the oosphere may escape beyond the collar whilst still within the cavity of the conceptacle. The swollen layer of wall serves to attach the oosphere to the tubular attaching stalk formed by the extended 'collar'. Antherozoids have been observed swimming in the region between wall and oosphere, and germinating sporelings are found *in situ*, so that, although fertilization has not actually been observed, it must occur whilst the oospheres are still attached to the conceptacle and are still surrounded by the swollen mucilaginous layer of wall.

The attaching stalks are very tenacious and, at the time of extrusion, the oospheres are firmly attached and can be teased apart with needles without becoming detached. The mucilage layer immediately surrounding the oosphere gradually swells in the sea-water and disintegrates, thus liberating the

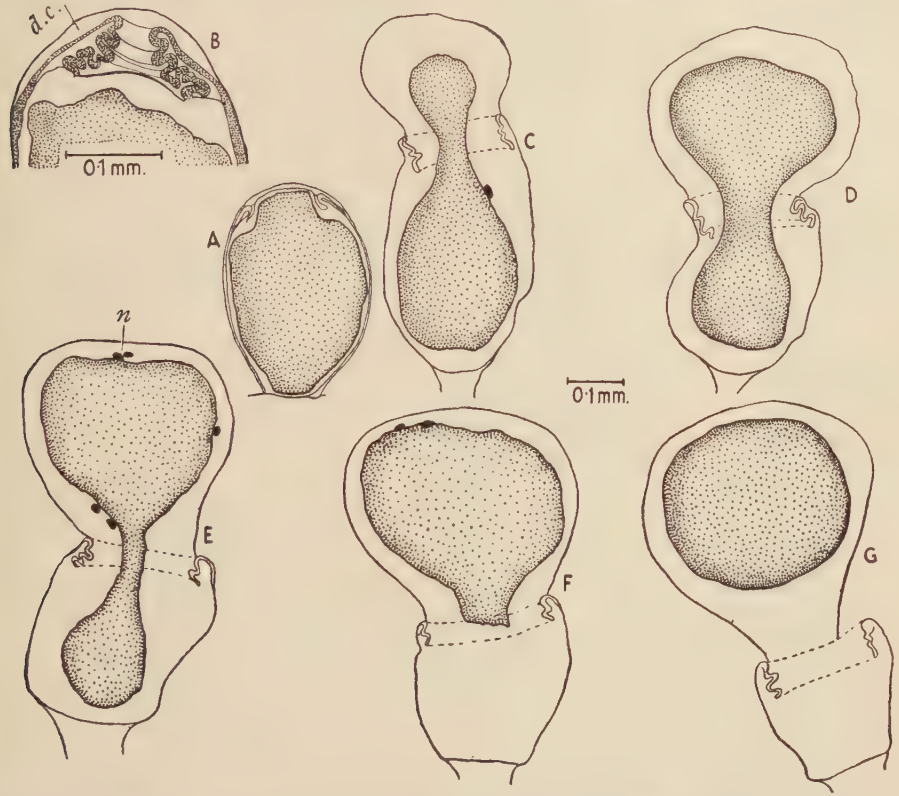

Fig. 9. Stages in the extrusion of the oosphere. A. Mature oogonium immediately before extrusion. B. Detail of distal region of oogonium after slight swelling in potash. The distal collar (*d.c.*) can be clearly seen to be continuous with the swollen layers of wall at its inner margin, and at its outer end to form a continuous layer between the exochite and the swollen layers. C–G. Stages outside the conceptacle showing the escape of the oosphere—still surrounded by the swollen middle layer—from the cup formed from the 'collar'. The top of the hollow, tubular stalk formed from the extended collar can be seen. In C, E, and F the extruded supernumerary nuclei (*n*) can be seen. With the exception of B, all these figures are drawn from living material mounted in sea-water. All outlines drawn with a camera lucida.

spores, which sink to the bottom, leaving tufts of stalks protruding from the ostiole. Although sporelings of a week old or more have been found still attached in cultures, it is improbable that they remain attached so long under natural conditions, as a slight jar easily detached them. Probably wave action would detach them after about 24 hours. The solid attaching stalks of *Carpophyllum maschalocarpum* are much more tenacious, and even after a week it is difficult to detach the young sporelings. The oospheres of *M. urvilliana* remain viable for about a week, after which they swell up and burst.

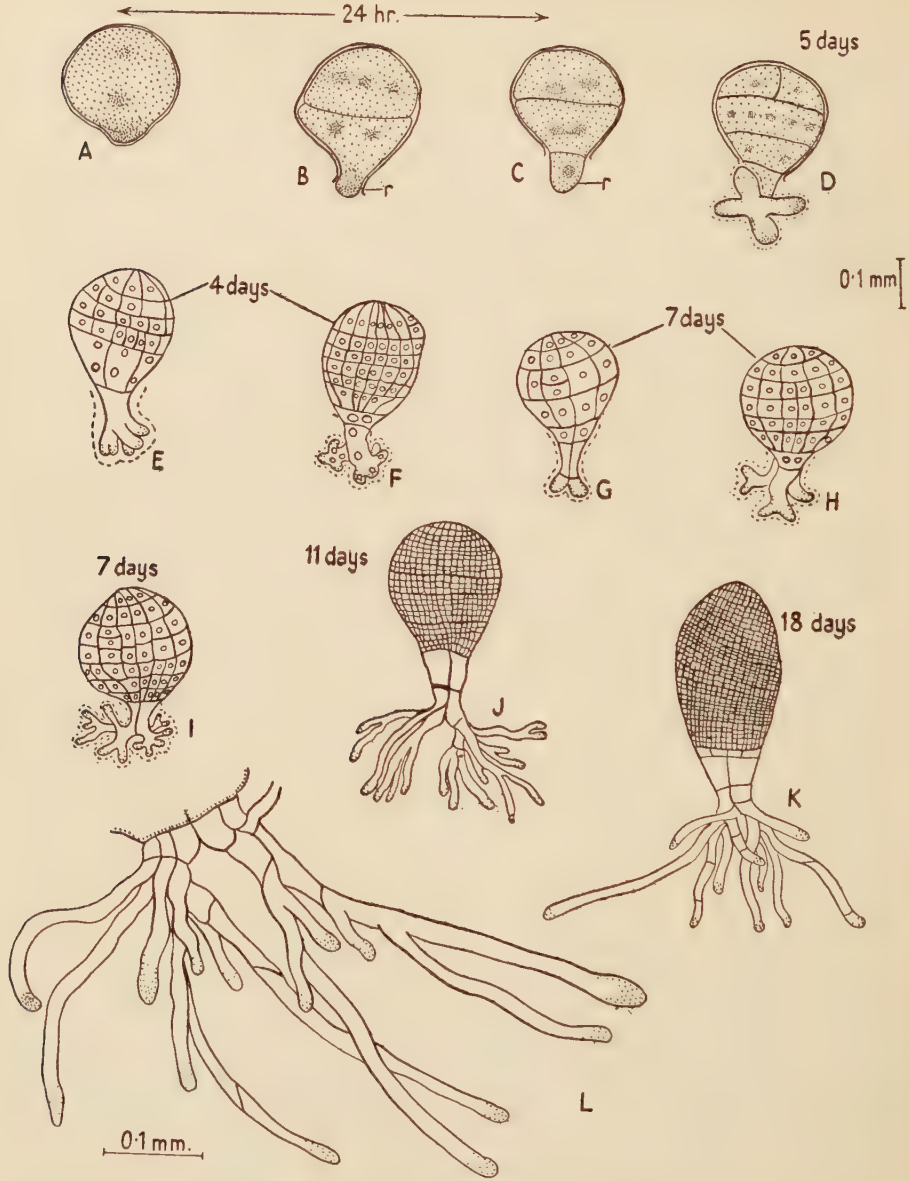

FIG. 10. Stages in the germination of the oosphere. In each case the age of the sporeling is indicated. A–K, the entire sporeling. L, the rhizoidal system at a higher magnification. *r* = primary rhizoid. All outlines drawn with a camera lucida.

Sporeling Development

Development of sporelings was followed in cultures for periods of up to 4 months, by which time they were about 2 mm. in length with a funnel-like apical depression. After this stage the sporelings began to show signs of starvation, and no further appreciable growth took place up to 6 months. Plants of a comparable age collected from the shore were heart-shaped plates about a centimetre long (Fig. 1, A).

The early stages of development took place *in situ* whilst still attached to the receptacles, the sporelings dropping off and sinking to the bottom of the dishes, where they became firmly attached at varying intervals after 24–48 hours and up to 1 week.

The oospore is surrounded by a firm wall, distinct from the mucilaginous layer of oogonium wall. Within 24 hours of liberation a protuberance develops

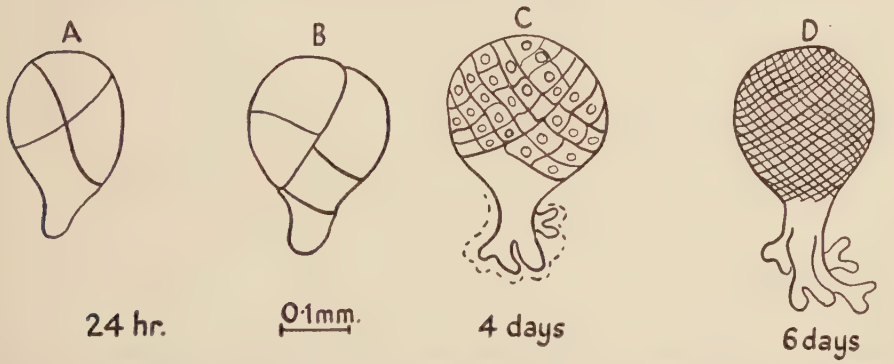

Fig. 11. Sporelings in which the first cleavage was oblique. Outlines drawn with a camera lucida.

towards one end of the oospore and there is an aggregation of denser cytoplasm in this region. This is accompanied by a nuclear division, and the first wall is formed, transversely and medianly. Further nuclear divisions follow, and the protuberance bursts through the oospore wall, before a second wall cuts off the primary rhizoid (Fig. 10, A–C). Further wall formation follows rapidly in the body portion of the sporeling, the upper cell becoming divided at an early stage into quadrants. For the first week or so—11 days in this case—no increase in size of the body was seen, cell divisions merely resulting in further subdivision of the previously existing cells, so that the surface layer consists of progressively smaller cells, in the arrangement of which the lines of the early segmentations can long be traced (Fig. 10, D–I). Occasionally (Fig. 11, A) the first cell-wall arises obliquely relative to the primary rhizoid. Such sporelings appear to develop quite normally, at least for 6 days, after which the line of the original segmentation could no longer be clearly distinguished.

After this period of subdivision an increase in size occurs, and at 18 days sporelings were found with elongated, ovate bodies with pointed tips (Fig.

10, K). At this stage they were still prostrate, but shortly afterwards became erect, and the pointed tip was replaced by a blunt, indented apex.

At an early stage the primary rhizoid divides into four short branches with characteristic bifurcated tips surrounded by deeply staining mucilage (Fig. 10, D). They grow rapidly and divide to form a complex system of apically growing attaching rhizoids, whose origin can long be traced to the first four segments of the primary rhizoid (Fig. 10, L). Occasionally a second series of rhizoids grows down from the cells immediately above the primary rhizoid.

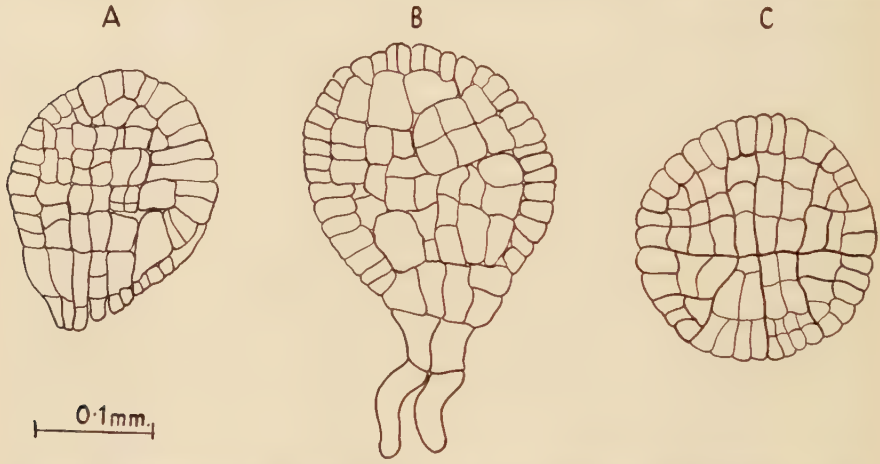

FIG. 12. Sections through 1-week-old sporelings still attached to the receptacle (cf. Fig. 10, G, H, I). A. Longitudinal section showing elongation of lower cells to form rhizoids. Meristoderm and medulla are already differentiated. B. Longitudinal section of slightly more developed sporeling than A. C. Transverse section, still showing the early lines of cleavage. Outlines drawn with a camera lucida.

Thus, in the early formation of a primary rhizoid before wall formation begins, *M. urvilliana* resembles *Fucus* and those members of the Fucaceae which have been described. The early subdivision of this primary rhizoid into four basic branches is unlike anything recorded in the Fucaceae, but resembles rather the Cystoseiraceae and Sargassaceae, although in these two families the subdivision takes place at an even earlier stage, so that a tuft of from 8 to 32 primary rhizoids is formed. Inoh (1930) correlates the number of primary rhizoids with oosphere and primary rhizoidal cell size.

Sections through week-old sporelings (Fig. 12, A–C) show that meristoderm is differentiated from the central tissues at an early stage, and as elongation of the sporeling commences the most central cells become elongated to form the medulla, leaving a narrow cortical region beneath the meristoderm.

Discussion

The Fucaceae and Cystoseiro-Sargassaceae are usually separated on grounds of:

1. *Symmetry*, the former being bilateral, the latter radial.

2. *The apical cell*, which is four-sided in adult plants of the former series, three-sided in the latter. It is lodged at the base of a depression, which is a groove elongated in the plane of flattening in the former, and a radially symmetrical pit or groove in the plane perpendicular to the plane of flattening in the latter.
3. *The nature of the medulla*, which was described by le Touzé (1912) as being compact and parenchymatous with little hypha and mucilage production in the latter series, but characterized by copious mucilage formation and abundant hypha production in the former.
4. *The pronounced tongue-cell* of the *Cystoseiro-Sargassaceae* (Schmidt, 1938).
5. *The germination of the oospore* to give a single rhizoidal initial in the first, and a tuft of 8–32 primary rhizoids in the latter series.

To these criteria, Dawson (1941) suggests the addition of:

6. The nature of the attaching stalk (if any) which is solid in the latter series, but hollow and tubular in the first.

Phyllospora, *Scytothalia*, *Seirococcus*, and *Marginariella* are four endemic southern genera whose morphological features indicate that they are closely related. Information about *Phyllospora* and *Seirococcus* is available from unpublished observations by A. E. E. Dawson and the author. All possess monopodial main axes from which arise distichous series of leafy laterals; all show characteristic incurving of the young laterals around the apex, where the apical cell is lodged at the base of a shallow groove orientated at right angles to the plane of flattening (cf. *Himanthalia*, *Halidrys*); all possess special adventitious receptacles which grow by an apical cell and probably represent modified lateral branches. They are unusual amongst the Fucaceae in their submerged habitat, in the much branched attaching organ, in the development of a single egg of characteristic amphora shape in each oogonium, and in its retention until after fertilization by a hollow stalk formed from the middle layers of the oogonium wall.

In both *Marginariella* and *Scytothalia* the tongue-cell divides to form an ectocarpoid hair, which persists until the conceptacle has reached an appreciable size. The condition in *Seirococcus* and *Phyllospora* is not known. The straight wall by which it is cut off in the first place resembles the condition in *Fucus* rather than the curved wall common in the Cystoseiro-Sargassaceae. Germination of the oospore of *Marginariella* resembles the Fucaceae in the formation of a single primary rhizoid; but its early subdivision is unusual. The manner of germination of the other three genera is not yet known.

These four genera resemble the Cystoseiro-Sargassaceae in the compact structure of their medulla, and at first were classified in this family. Grüber (1898), however, examined in detail the apical cell of *Seirococcus* and found it to be four-sided, as was that of *Phyllospora* and *Scytothalia*, and so he transferred this series to the Fucaceae. I have recently been able to confirm the four-sided nature of the apical cell in the last two genera. He did not,

however, have suitable apices of *Marginariella* to section, but placed it in the Fucaceae with the other three genera on grounds of similarity of external features of the apex and general morphology. Baker (1950) has recently shown that in salt-marsh forms of several species of British members of the Fucaceae the apical cell may be three-sided. So the shape of the apical cell may be more variable than was previously realized, and possibly the possession of a three-sided apical cell is not a feature of sufficient weight to warrant the separation of *Marginariella* from the three genera which it so closely resembles in many other ways.

The affinities of *Marginariella* are thus clearly with *Scytothalia*, *Phyllospora*, and *Seirococcus*, and together these form a rather isolated group, having some features in common with the Fucaceae and some with the Sargassaceae. On the whole, however, the balance appears to be with the Fucaceae on grounds of symmetry, shape of apical cell, the cutting off of the tongue-cell by a straight wall, and the mode of germination of the oospore.

Fritsch (1945) regards them as being the most highly specialized members of a series the beginnings of which are seen in the British *Ascophyllum* and the South African *Axillaria*.

Possibly, however, the branched attaching organ, the characteristic form of the oosphere, and the modification of the distal portion of the oogonium wall to form the tubular attaching stalk are features of sufficient weight to warrant the separation of this group of genera into a distinct series.

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